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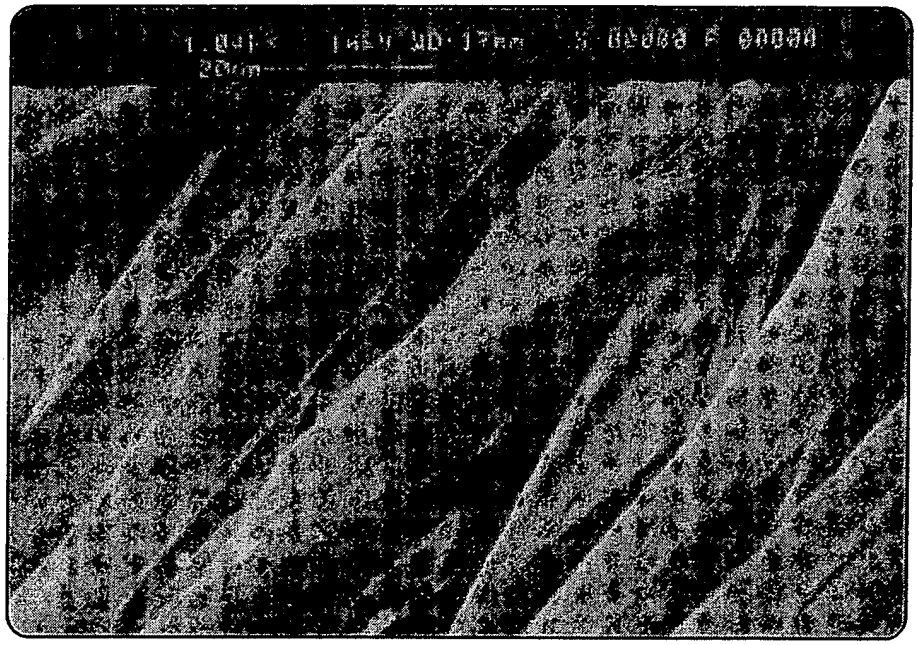

Lea Heikinheimo

Trichoderma reesei cellulases in processing of cotton

VTT PUBLICATIONS 483

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Lea Heikinheimo

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Abstract

Today the use of enzymes in textile processing and after-care is already well established industrial technology. Enzymatic process applications have increased substantially due to developments in genetic engineering, as specific enzymes can be efficiently modified for targeted applications. In addition, being biological molecules and efficient catalysts, enzymes can provide environmentally acceptable routes to replace harsh chemicals. Furthermore enzymatic processes can be applied using equipment already existing in the textile industry.

The cellulases of the soft-rot fungus *Trichoderma reesei* are the most studied and understood of all cellulolytic systems. Cellulases are used for modification of cellulosic fibres and fabrics, e.g. cotton, viscose and lyocell, yielding properties such as stonewashing, peach-skin and biofinishing effects. Cellulases are usually applied as multi-component enzyme systems and most of the commercial cellulases contain a variety of different activities. The cellulolytic system of *T. reesei* is composed of two cellobiohydrolases (CBHI and CBHII), at least six endoglucanases and two β-glucosidases. Cellulases are known to act synergistically in the hydrolysis of crystalline cellulose. Endoglucanases randomly attack the amorphous regions in cellulosic substrates, whereas cellobiohydrolases can also act the crystalline regions of cellulose, releasing cellobiose from the ends of cellulose chain.

In the present investigation, purified *T. reesei* cellulases CBHI, CBHII, EGI and EG II were used to treat different types of cotton fabrics in order to evaluate the effects of individual mono-component cellulases on cotton properties. By comparing the impact of mono-component cellulases on cotton twill and poplin woven fabrics and interlock knitted fabric, it became apparent that cellobiohydrolases and endoglucanases have different effects on the tested fabrics. CBHII did not have any pronounced effect on cotton. By contrast CBHI

produced significant amounts of reducing sugars and caused weight loss of fabrics. When a high hydrolysis degree was used, i.e. the weight loss was pronounced, EGII caused more strength loss than either EGI or CBHs. By limiting the treatment time and using additional mechanical action it was observed that EGII was able to improve the pilling properties of cotton fabrics even at low weight and strength loss levels.

In addition, the possible synergistic effects between different cellulases were evaluated with different ratios of endo- and exoglucanases. According to weight loss and reducing sugar analyses, both endoglucanases exhibited clear synergism with CBHI. EGI also showed slight synergism with CBHII. Practically no endo-endo or exo-exo synergism was observed on the basis of weight loss analysis. Compared to cellulase mixtures, the EGII treatment alone improved the pilling resistance more and resulted in less weight and strength losses at the same protein dosages. Thus, there was no correlation between high weight loss and good pilling results.

On the basis of the knowledge obtained from the mono-component treatments, new cellulase preparations with different profiles of *T. reesei* cellulases were developed. Using these experimental cellulases, it was found that high pilling removal was dependent on the fabric type, and again EGII-based cellulase products yielded the most positive depilling results. It was also shown that the strength loss could be minimized by having only EGII present in the cellulase mixture.

The effects of the purified endoglucanases and cellobiohydrolases and an experimental cellulase mixture on denim were also evaluated. The results confirmed that endoglucanases are the cellulases required for a good stone washing effect, and EGII was the most effective in removing color from denim despite a very low hydrolysis level. CBHI did not produce any stone washing effect.

When the impact of purified cellulases on the molecular weight distribution of cotton powder obtained after enzyme treatment was studied, EGII was the only enzyme which reduced the molecular weight of cotton powder with high mechanical action. The results also showed that mechanical agitation affected the performance of EGII more than that of EGI measured as weight loss and molecular weight of cotton powder.

Preface

This work was carried out at VTT Biotechnology during the years 1996 – 2002. I thank Professor Liisa Viikari for providing excellent working facilities. I also express my warm thanks to Professor Pertti Nousiainen from the Institute of Fibre Materials Science of Tampere University of Technology for his support during my studies. I am grateful to Professor Jorma Sundquist and Dr. Georg Gübitz for useful comments and critical reading of the thesis.

I warmly thank the personnel of the Biotechnical Laboratory for a friendly and supporting atmosphere. I express my sincere thanks to my instructor Dr. Johanna Buchert for introducing me to the fascinating world of biochemistry and for her support, ideas and constructive criticism during these years. My very special thanks are due to Kati Uotila for her excellent technical assistance and positive attitude. I also thank my other co-authors at VTT, Arja Miettinen-Oinonen and Matti Siika-aho for all their help, discussions, critical reading of this thesis and for providing the purified and the experimental enzymes. I am very grateful to Michael Bailey for revising the English language of the manuscripts and of the thesis and Oili Lappalainen for helping in preparing the figures.

I express my gratitude to the personnel of the Institute of Fibre Materials Science of Tampere University of Technology for providing excellent working facilities and a friendly atmosphere. My most sincere thanks are due to Arja Puolakka, Marianne Vehviläinen, Maritta Suoniemi and Raimo Haapaniemi for their useful help and support throughout my studies. I also thank everyone at CITEVE, especially my other co-authors Professor Luis Almeida, Jose Morgado and Professor Artur Cavaco-Paulo from University of Minho for making my stay in Minho, Portugal pleasant. The financial support of the Finnish Cultural Foundation, Federation of Finnish Textile and Clothing Industries and Leonardo da Vinci program and Lahti Polytechnic is gratefully acknowledged.

Finally, I express my warmest thanks to my husband Jouni and our daughters Lilli and Anni for their love, patience and support throughout this work. I also thank my parents Kaisa and Eino, Jouni's parents, Anna-Maija and Jaakko and my sisters Anne and Pia and our friend Jonna for helping me in so many ways, especially in taking care of Lilli and Anni.

List of original publications

This work is based on the following articles, referred to in the text by the Roman numerals given below. Additional unpublished data is also presented.

I Heikinheimo, L., Cavaco-Paulo, A., Nousiainen, P., Siika-aho, M. and Buchert, J. 1998. Treatment of cotton fabrics with purified *Trichoderma reesei* cellulases. JSDC 114 July/August, 18–22.

II Heikinheimo, L. and Buchert, J. 2001. Synergistic effects of *Trichoderma reesei* cellulases on the properties of knitted cotton fabric. Textile Res. J. 71(8), 672–677.

III Heikinheimo, L., Miettinen-Oinonen, A., Suominen, P. and Buchert, J. 2000. Treating denim fabrics with *Trichoderma Reesei* Cellulases. Textile Res. J. 70(11), 969–973.

IV Miettinen-Oinonen, A., Heikinheimo, L., Buchert, J., Morgado, J., Almeida, L., Ojapalo, P. and Cavaco-Paulo, A. 2001. The role of *Trichoderma reesei* cellulases in cotton finishing. AATCC Review, January, 33–35.

V Heikinheimo, L., Miettinen-Oinonen, A., Cavaco-Paulo, A. and Buchert, J. 2002. Effect of purified *Trichoderma reesei* cellulases on formation of cotton powder from cotton fabric. Submitted to J. Appl. Polym. Sci.

The author of the present thesis had main responsibility for the practical work in all publications, except the development of experimental cellulase mixtures in publications III and IV which was carried out by Arja Miettinen-Oinonen.

Contents

Appendices of this publication are not included in the PDF version. Please order the printed version to get the complete publication (http://otatrip.hut.fi/vtt/jure/index.html)

Abbreviations

CBD	cellulose binding domain
CBH	cellobiohydrolase
DP	degree of polymerization
d.w.	dry weight
EG	endoglucanase
egl	gene encoding EG
GPC	gel permeation chromatography
HPLC	high performance liquid chromatography
L-value	lightness as reflectance unit
MW	molecular weight
pI	isoelectric point
RH	relative humidity
SEC	size exclusion chromatography
SEM	scanning electron microscope
TC	total crude cellulase

1. Introduction

The use of enzymes in textile processing and after-care is already established industrial technology. Enzyme treatments of textiles, typically cellulose materials such as cotton, viscose or lyocell fabrics, have widely been used in the textile industry since the 1980s, when the usefulness of enzymes for the replacement of pumice stones in stonewashing of denim fabrics and to soften cotton garments was discovered. Currently textile enzymes have a market value of US $ 200 million, equivalent to 12% of the industrial enzyme market (Ojapalo, 2002).

Enzymatic processing enables the textile industry to reduce production costs, to reduce the environmental impact of the overall process and to improve the quality and functionality of the final products. Enzymes are non-toxic and they allow mild conditions of temperature and pH. Today enzymatic treatment of cotton either in denim washing or in biostoning is standard technique in industry. Although the use of cellulases in cotton finishing has been increasing very rapidly, the cellulases used hitherto have mainly been crude mixtures causing unacceptable losses of fabric strength and weight. Furthermore, the unoptimized cellulase composition of commercial preparations and non-optimal dosage of the enzymes have lead to low reproducibility of the processes.

Cotton is still the most important of all textile fibres, accounting for 36% of textile fibre production (Table 1). Of the synthetic fibres, polyester is by far the most widely used fibre. Today it accounts for 30% of textile fibre production and its production rate is still expected to grow. The share of man-made cellulosic fibres e.g. viscose, modal, cupro, lyocell, acetate and triacetate, has declined since the 1960s, being today about 5% of the total textile fibre production (Table 1).

Table 1. World production of cotton, wool and man-made fibres in 2000 (CIRFS, 2001).

			Man-made fibres			TOTAL
	Raw Cotton	Raw Wool	Synthetics	Cellulosics	Total	
1000 tonnes	18 901	1390	29 970	2762	32 732	53 023
in %	35.5	3	56.5	5	62	100

1.1 Structure of cotton

Cotton, the seed hair of plants of the genus *Gossypium,* is the purest form of cellulose readily available in nature. It has many desirable fibre properties making it an important fibre for textile applications. Cotton has high moisture regain, moderate strength, and is stronger when wet (Table 2). However, it is not abrasion- or wrinkle-resistant.

Table 2. Properties of cotton (Billmeyer, 1984; Boncamper, 1999).

Property	**Cotton**
Fibre length (mm)	12–64
Fibre diameter (μm)	12–22
Tenacity, dry (g/den)	2.1–6.3
Tenacity, wet (g/den)	2.5–7.6
Elongation (% at break)	3–10
Specific gravity (g/cm^3)	1.50
Moisture regain, 21°C, 65% RH	8.5

Cotton fibre has a fibrillar structure and it consists of a primary wall, a secondary wall and a lumen (Fig. 1) (Nevell, 1995). In addition to cellulose, cotton also contains lipids, proteins, pectins, waxes, organic acids and non-cellulosic polysaccharides constituting up to 10% of the total fibre weight (Hartzell and Hsieh, 1998; Buchert *et al.*, 2000). These components are mainly located on the primary wall of the fibres. The secondary wall constitutes the bulk of a mature fibre and consists almost entirely of fibrils of cellulose arranged spirally around the fibre axis, the direction of the spiral reversing many times along a single fibre (Figure 1.).

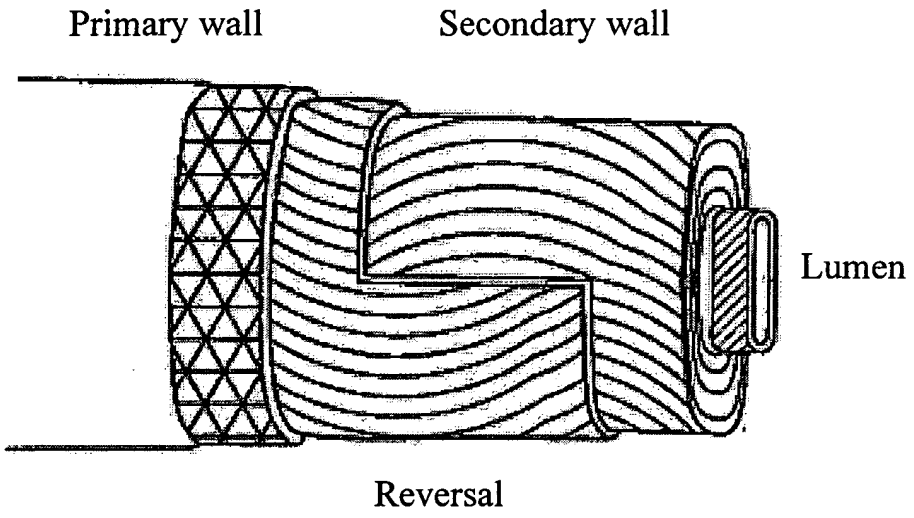


Figure 1. Structure of cotton (Nevell, 1995).

Cotton cellulose consists of crystalline fibrils varying in complexity and length and connected by less organized amorphous regions with an average ratio of about two-thirds crystalline and one-third non-crystalline material, depending on the method of determination (Morton and Hearle, 1997). The crystalline part of cellulose can occur in different crystalline lattice types, called cellulose I, cellulose II, cellulose III, cellulose IV or cellulose X, but only cellulose I and cellulose II are important in textile processing (Nevell, 1995). The form that naturally occurs in nature is cellulose I. Cellulose II is the thermodynamically stable form produced when cellulose I is destroyed by swelling with strong

alkali (mercerisation) or regenerated from solution in the viscose process (Engelhardt, 1995; Inglesby and Zeronian, 1996; Lenz *et al.*, 1990).

The chemical composition of cellulose is simple, consisting of anhydroglucose units joined by β-1,4-glucosidic bonds to form linear polymeric chains (Fig. 2). The chain length, or degree of polymerisation (DP), of a cotton cellulose molecule represents the number of anhydroglucose units connected together to form the chain molecule. DP of cotton may be as high as 14 000, but it can be easily reduced to 1000–2000 by different purification treatments with alkali (Nevell, 1995). According to Battista (1950), the crystalline regions probably have a DP of 200 to 300. Correspondingly, the molecular weight (MW) of cotton usually lies in the range of 50 000–1 500 000 (Sundquist, 1983) depending on the source of the cellulose. The individual chains adhere to each other along their lengths by hydrogen bonding and van der Waals forces. The physical properties of the cotton fibre as a textile material, as well as its chemical behaviour and reactivity, are determined by arrangements of the cellulose molecules with respect to each other and to the fibre axis.

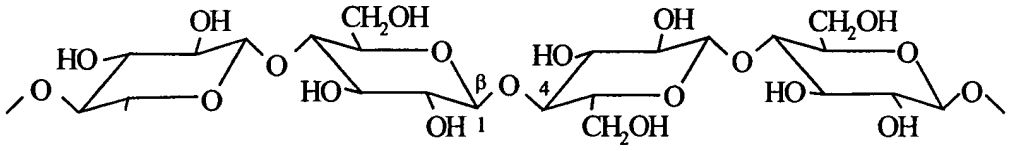


Figure 2. Structure of cellulose.

1.2 Enzymatic degradation of cellulose

In nature cellulose is degraded by both fungi and bacteria. These organisms produce cellulases that specifically degrade cellulose, yielding shorter chain cellulose polymers and glucose which are metabolised by these organisms. Typically, the fungal and some bacterial cellulolytic enzyme systems consist of several enzymes acting at the ends (exoglucanases, also called cellobiohydrolases) or in the middle (endoglucanases) of the cellulose chains (Wood, 1992). *Trichoderma reesei* is one of the most important industrially used strains for cellulase production. According to current knowledge, its cellulolytic

system is composed of two cellobiohydrolases (CBHI and CBHII) and at least six endoglucanases (EGI, EGII, EGIII, EGIV, EGV and EGVI) and two β-glucosidases (Srisodsuk, 1994; Saloheimo *et al.*, 1997, 2002). The sixth endoglucanase, named EGVI, has been described on the protein level (Bower *et al.*, 1998).

The endoglucanases mainly hydrolyse internal bonds in the cellulose polymer, producing new chain ends and thereby causing a considerable decrease in cellulose DP (Wood, 1989). Exoglucanases initiate the hydrolysis at the chain ends, and do not produce significant amounts of new chain ends on the cellulose surface (Irwin *et al.*, 1993). CBHII splits cellobiose from the non-reducing and CBH I from the reducing ends of cellulose chains (Vrsanská and Biely, 1993; Barr *et al.*, 1996). Cellobiohydrolases can also act on crystalline cellulose without the aid of endoglucanases (Chanzy and Henrissat, 1985). β-glucosidases complete the hydrolysis process by catalyzing the hydrolysis of cellobiose to glucose (Figure 3). Efficient overall hydrolysis of crystalline cellulose by cellulases requires the synergistic action of both EGs and CBHs, as reviewed by Teeri and Koivula (1995). Maximum synergism is usually obtained with a large amount of exo-enzyme and a minor amount of endo-enzyme (Reinikainen, 1994; Bailey *et al.*, 1993). It is also known that the degree of synergy is dependent on the substrate used (Nidetzky *et al.*, 1993).

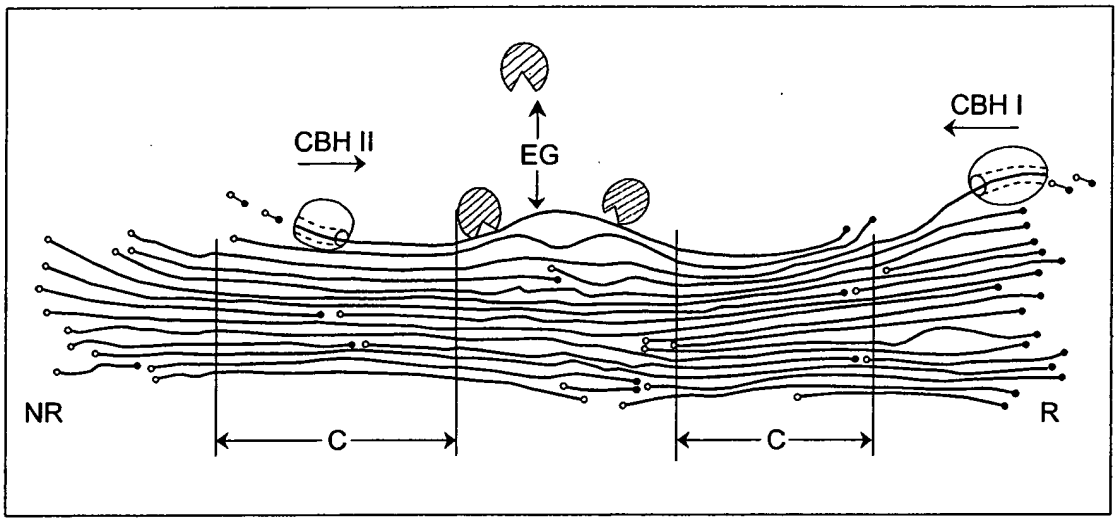
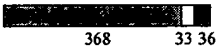
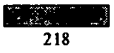
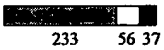
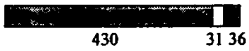
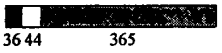


Figure 3. Mechanism for enzymatic hydrolysis of cellulose (Teeri, 1997). C defines the crystallinen regions. The filled circles, denoted R, represent the reducing ends and the open circles, denoted NR, represent the non-reducing ends.

Most fungal cellulases consist of two domains, i.e. a larger catalytic domain and a smaller cellulose binding domain (CBD). These domains are joined by a glycosylated linker peptide (Tomme *et al.*, 1988; Teeri *et al.*, 1992). Of the identified *T. reesei* cellulases only EGIII lacks the CBD and linker region (Table 3). The catalytic domain contains an active site with the shape of a "tunnel" or an open cleft in the case of CBHs and EGs respectively. The diameter of the catalytic cores of CBHs is 60 to 64 Å and the length of the whole enzyme is about 180 Å for CBHI and 210 Å for CBHII (Reinikainen, 1994). The presence of CBD is essential to the degradation of solid crystalline cellulose (Tomme *et al.*, 1988; Srisodsuk *et al.*, 1993). Many studies have shown that removal of the CBD typically results in a decrease of about 50–80% of the activity of fungal cellulases on insoluble but not on soluble substrates (van Tilbeurgh *et al.*, 1986; Tomme *et al.*, 1988; Reinikainen *et al.*, 1995; Suurnäkki *et al.*, 2000). The loss of hydrolytic ability with the loss of CBD is especially pronounced on crystalline cellulose (Suurnäkki *et al.*, 2000).

Cellulases have been classified into families sharing similar structures and the same chemical reaction mechanism by sequence and structural comparison. Hitherto, more than 180 different CBDs have been identified and classified into 13 different families according to their amino acid sequence similarities (Tomme *et al.*, 1995). Recently, a new nomenclature of cellulases based solely on glycoside hydrolase families has been proposed (Henrissat *et al.*, 1998).

Table 3. Properties and schematic structural organisation of cellulases from T. reesei (Srisodsuk, 1994; Saloheimo et al., 1997).

Enzyme	Family	Amino acid residues	Molecular mass kDa	Isoelectric point (pI)	Structural organization[b]
EGI	7	437	50–55	4.6	
EGII	5	397	48	5.5	
EGIII	12	218	25	7.4	
EGIV	61	326	(37)[a]	–	
EGV	45	225	(23)[a]	2.8–3	
EGVI	unknown*	unknown*	95–105	5.6–6.8	unknown*
CBHI	7	497	59–68	3.5–4.2	
CBHII	6	447	50–58	5.1–6.3	

a The molecular mass calculated from amino acid sequence
b ■ , the catalytic domain; □, linker region; ■ , CBD
* gene not described

1.2.1 Cellulase activity measurements

The comparison of cellulase mixtures or individual cellulases poses special problems related to the multicomponent nature of the enzymes and their different efficacies on different cellulose structures. A wide range of different model substrates and many different assays have been developed and used to demonstrate the specific action of cellulases. Examples of methods for measuring cellulolytic activity have been summarized by Reinikainen (1994).

Cellulase activity measurements can be divided into two groups: one type using soluble substrate and the other using insoluble substrate such as cotton, dyed Avicel or filter paper. When using soluble substrate, such as carboxymethyl cellulose (CMC) or hydroxyethyl cellulose (HEC), the amount of reducing sugars liberated or the reduction in viscosity as a function of time can be measured. These methods are interpreted as endoglucanase activity measurements because the presence of side groups in CMC and HEC appears to prevent the action of most CBH enzymes produced by fungal systems on these substrates (Wood, 1992). EGI and EGII, the major endoglucanases, exhibit marked activity on β-glucan and soluble cellulose derivatives, such as HEC (Suurnäkki *et al.*, 2000) and they are therefore considered to be true endoglucanases (Reinikainen, 1994).

For the determination of total cellulolytic activities of cellulase mixtures or their complex synergistic action, more heterogeneous substrates such as filter paper, Avicel and cotton can be used. As a model substrate, highly crystalline, bacterial microcrystalline cellulose (BMCC) with a simple morphology has been used to study crystal erosion (Reinikainen, 1994).

When evaluating cellulases designed for the textile industry, the comparisons should not be based on assays run on soluble substrate, but on the effect on a relevant cotton fabric (Videbaek *et al.*, 1993; Cavaco-Paulo *et al.*, 1996b). Although the main priority of textile chemists is to identify the activities which deliver certain finishing effects, it is also essential to understand the mechanism of hydrolysis and how mechanical action affects the liberation of reaction products.

1.3 Use of enzymes in processing of cellulosic textile materials

Enzymes have been used as process aids in the textile industry for decades. Desizing with amylases was the first applications of enzymes (Cavaco-Paulo, 1998b). With the development of the application of cellulases for treatment of denim and other cotton fabrics the textile industry became a focus area for the enzyme manufacturers. Today approximately 80% of all denim is treated with cellulases. In addition, about 10% of all other finishing of cellulose materials is

now performed using cellulases (Buchert and Heikinheimo, 1998). An overview of the application of cellulases and other enzymes studied or used for textiles are listed in Table 4.

Table 4. An overview of enzymatic textile applications investigated and their commercial status.

Type of fiber	Enzyme substrate	Enzyme	Application	Commercial status	Ref.
	Starch	A-amylases	Desizing	C	Cegarra, 1996
Cotton	Pectin	Pectinase	Bioscouring	C/P	Takagishi, *et al.*, 2001
	Protein	Proteases	Bioscouring	R	Hsieh and Cram, 1999; Buchert *et al.*, 2000
	Cellulose	Cellulases	Biostoning	C	Tyndall, 1990; Kochavi *et. al.*, 1990
			Biofinishing	C	Tyndall, 1992
	Indigo	Laccase /mediator	Decolorization of indigo	C/P	Vollmond, 1997
Lyocell, Viscose, Modal	Cellulose	Cellulases	Biofinishing, peach skin effect	C C	Kumar *et al.*, 1996; Kumar and Harnden 1999
	Hydrogen peroxide	Catalase	Removal of H_2O_2 after bleaching	C	Schmidt, 1995; Jensen, 2000
Wool	Protein	Proteases	Shrinkage reduction, improved dyeability, hand and whiteness	C, R	Chikkodi, 1996; Yoon *et al.*, 1996; Nolte *et al.*, 1996; Hughes *et al.*, 2001; Miettinen-Oinonen *et al.*, 2001; Schumacher *et al.*, 2001
	Cellulose impurities	Cellulases	Carbonisation	R	Fornelli 1992a; Byrne and Rigby, 1995

continues

Table 4. continued

Silk	Protein	Proteases	Degumming, sand washed effect	C	Byrne *and* Rigby, 1995
Acrylic	Nitrile	Nitrile hydratases and nitrilases	Improvement of dyeing	R	Tauber *et al.*, 2000
Polyester	BEB, ETE*	Esterases	Depilling and improvement of dyeing	R	Andersen *et al.*, 1998
Dissolving pulp	Cellulose and hemicellulose	Cellulases and hemicellulases	Production of regenerated fibers	R	Rahkamo *et al.*, 1996, 1998; Vehviläinen *et al.*, 1996; Struszczyk *et al.*, 1995

Commercial status: research (R), pilot (P) or commercial (C)
*BEB: ethyleneglycol dibenzyl ester, ETE: terephtalic acid diethyl ester
(For commercial products, see also: www.novozymes.com, www.abenzymes.com, www.genencor.com)

1.3.1 Desizing with amylases

Starch and its derivatives are the sizes most widely used for coating of warp yarns in order to prevent the threads from breaking during weaving. Starch is a cheap, natural raw material and it is biodegradable. Starch is a mixture of two polysaccharides, amylase and amylopectin consisting mainly of α-1,4-linked glucose units (Fornelli, 1992b). After weaving the size must be removed since sized fabrics are less absorbent especially for dyes. Desizing can be carried out by prolonged cooking or by using strong chemicals such as acids, bases or oxidizing agents.

Enzymatic treatment with amylase enzymes has replaced the harsh processes since the beginning of the last century. The product range of amylases includes mesophilic and thermophilic enzymes allowing desizing at 20–115°C. Several applications can be used for desizing, such as padding, jigger and continuous

high speed processes, in which the reaction time for the enzyme may be as little as 15 seconds (Novozymes, 2002). However, there is still considerable scope for improving the speed, economics and consistency of the process, including the development of more heat-stable enzymes, optimization of BOD levels, as well as a better understanding of how to characterise their activity and performance with respect to different fabrics, sizes and processing conditions (Cegarra, 1996). Many commercial α-amylases are available and it is estimated that approximately 15% of all commercial textile enzymes are used in desizing processes (Ojapalo, 2002).

1.3.2 Enzymes in processing of cotton

The greatest number of enzymatic treatments has been applied to industrial processing of cellulosic fibres in order to obtain new finishing effects or to replace harsh chemicals used in conventional cotton processes. Conventional industrial processing of cotton may include several chemical steps performed during wet process stages as shown in Figure 4. Enzymatic treatments of cotton, subdivided into three categories i.e. biopreparation, biofinishing and biostoning, can be included in these wet processes and they can be performed in already existing equipment (Figure 4).

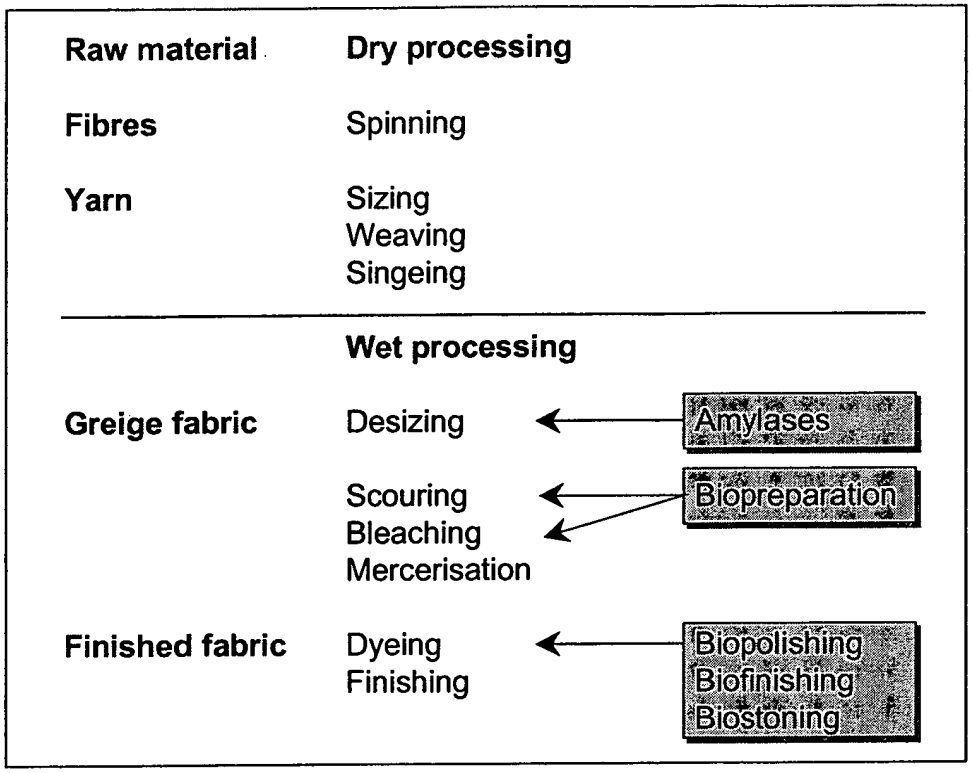


Figure 4. Stages of cotton processing.

1.3.2.1 Enzymes in biopreparation

At present conventional preparation methods, i.e. scouring and bleaching of cotton fabrics, involve the use of high concentrations of alkali, hydrogen peroxide and other chemicals, often combined with high temperatures, to ensure efficient removal of impurities prior to dyeing. These wet processes can cause depolymerization of cellulose molecules and significant losses in fabric weight and tensile strength (Buschle-Diller *et al.*, 2001; Li and Hardin, 1998). Furthermore, large quantities of water and energy are used and the alkaline effluents require special treatment. According to Rössner (1993), about 75% of the organic pollutant level arising from textile finishing is derived from the preparation of cotton goods.

Investigators at several centres have shown that impurities in cotton can be removed by specific enzymes (Waddell, 2002; Buchert *et al.*, 2000; Hartzell and Hsieh, 1998; Rössner, 1993). The removal of impurities of cotton, i.e. pectins, proteins and waxes has been investigated using different pectinases, proteinases, cellulases and lipases in order to increase the wettability and increase the whiteness of the fabric (Rössner, 1993; Buchert *et al.*, 2000; Buschle-Diller *et al.*, 2001; Lin and Hsieh, 2001). Enzymatic biopreparation of cotton represents a rather new approach and is still mainly in the development stages. A prototype continuous scouring machine has already been developed (Takagishi *et al.*, 2001). Cellulases have been reported to be the most efficient in improving wettability of cotton (Hartzell and Hsieh, 1998), and cellulase treatment applied before alkaline scouring has been shown to improve the removal of seed-coat fragments from desized cotton (Csiszár *et al.*, 1998). Enzymatic bleaching of cotton fabric with glucose oxidase, producing H_2O_2, has been investigated by Buschle-Diller *et al.* (2001). They established a process allowing the combination of enzymatic desizing, bioscouring and enzymatic bleaching with glucose oxidase. According to their results, the process yielded products with acceptable whiteness very close to that of commercially bleached goods, and excellent mechanical properties.

Many investigations have shown that pectinases and proteinases can be effectively used for preparing greige cotton for subsequent chemical treatments (Buchert *et al.*, 2000; Lin and Hsieh, 2001; Takagishi *et al.*, 2001; Yachmenev *et al.*, 2001). The removal of pectins and proteins was reported to improve the water absorbency of cotton fabric (Buchert *et al.*, 2000; Lin and Hsieh, 2001; Hsieh and Cram, 1999). Furthermore, alkaline pectinase treatment had no negative impact on the dyeing properties of cotton fabric (Etters *et al.*, 2001).

The use of catalase enzymes to break down residual hydrogen peroxide after bleaching cotton is an already established application (Weible, 1991). The catalase enzyme decomposes two moles of hydrogen peroxide into two moles of water and one mole of oxygen. The high rate of enzymatic degradation of hydrogen peroxide allows reduction of the water consumption during washing of the bleached cotton, and prevents problems in further dyeing (Schmidt, 1995; Jensen, 2000; Tzanov *et al.*, 2001; Costa *et al.*, 2001). Reactive dyes are especially sensitive to peroxides and currently require extended rinsing and/or use of reducing agents.

1.3.2.2 Enzymes in denim washing

Indigo-dyed denim jeans are the most popular items by far which are garment processed and approximately one billion pairs of jeans are produced per annum (McCarthy, 1999). Traditionally, denim has been woven with cotton in a warp face twill weave, in which the warp is blue whereas the weft is white. Dyeing is generally performed with pure indigo, or indigo combined with a sulphur dye to decrease the costs caused by the more expensive indigo dye (Etters, 1993). Indigo dye is popular because it washes down to clear bright blue shades without staining of the white weft yarns (Olson, 1988).

Denim jeans manufactures have washed their garments for many years with pumice stones to achieve a soft-hand as well as a desirable bleached-out character. Stone washing involves adding 1–2 kilograms of pumice stones per pair of jeans during wet processing and this causes significant processing and equipment problems. The main disadvantages of this procedure are the removal of the high loading of pumice from processed clothing items and from large tub equipment, which can easily be damaged by an overload of tumbling stones or the occurrence of pumice dust (Heine and Höcker, 1995). In view of the problems of pumice in stone washing, increasing attention was directed to finding a replacement for stone washing in garment manufacture. Cellulases have been used in denim washing since the 1980s (Tyndall, 1990; Kochavi *et. al.*, 1990) and it has been estimated that about 80% of denim washing is nowadays carried out using cellulases (Buchert and Heikinheimo, 1998). Cellulases are added to desized denim in a washing machine, and the combination of enzyme action and fabric/fabric friction, as well as abrasion by the machine, causes the desired fading and softening of the garment. Zeyer *et al.* (1994) drew up an empirical model of the decolorisation of cellulose fabrics. They concluded that mechanical action opens the outermost layers of the cellulosic crystal, thus increasing the part of the cellulose accessible to enzymes and allowing enzymatic removal of the dye. When using cellulases there is no need for time consuming and expensive removal of stone particles from the garments after processing. Machinery capacity can also be improved due to reduced processing times (Kochavi *et al.*, 1990).

During the cellulase treatment of denim, the indigo removed can redeposit or backstain the reverse or front sides of the fabric, thus reducing the desired

contrast between white and the indigo dyed fibres. Many studies have been made to optimize denim washing processes to obtain minimal backstaining and to understand the mechanism of backstaining (Kochavi *et al.*, 1990; Klahorst *et al.*, 1992; Ehret, 1994; Cavaco-Paulo *et al.*, 1998a). The effects of posterior washing of denim and application of an adequate agent during washing to avoid or minimize backstaining have also been investigated (Ehret, 1994).

First it was suggested that pH and time were the key factors in preventing backstaining, and therefore neutral cellulases (optimum at pH 7), e.g. from *Humicola insolens,* would cause less backstaining than acid cellulases (optimum at pH 5) (Kochavi *et al.*, 1990; Asferg, 1991). Lantto *et al.* (1996) showed that backstaining can occur at both acidic and neutral pH depending on the preparation used. However, it has been suggested that backstaining is caused by adsorption of dyes onto cellulase components bound to the fibres (Clarkson *et al.*, 1993). By adding proteinases during the cellulase wash or rinse step, reduction of backstaining can be obtained by removing or preventing the cellulase proteins from binding the coloured particles back onto the surface of denim (Clarkson *et al.*, 1993; Cavaco-Paulo *et al.*, 1998a; Yoon *et al.*, 2000). The influence of the CBDs on indigo backstaining has also been studied by Andreaus *et al.* (2000) and Cavaco-Paulo *et al.* (1999). Andreaus *et al.* (2000) showed that deletion of the cellulose binding domains from either bacterial or fungal cellulases decreased indigo backstaining levels more than the entire enzyme. This observation could be useful, if the cellulases could maintain their cellulolytic activity without CBDs.

Cavaco-Paulo *et al.* (1999) also studied the interaction of cotton fabric with CBD peptides of *Cellumonas fimi.* The results showed that the family II CBDs bound to cotton cellulose increased acid dye affinity, but caused very poor washing fastness values.

Several studies, listed in Table 5, have been made with CBH- and EG-rich components and several compositions have been suggested for better washing effects of denim.

Table 5. An overview of the patents and research on denim with modified or purified cellulases.

Enzymes used	Analyses	Results	Ref.
Trichoderma longibranchiatum free of CBH type components	Evaluating stone washing appearance	Reduced backstaining	Clarkson *et al.*, 1992b, WO 94/07983
T. reesei TC* + EGI, EGII, CBHI and CBHII	Weight loss, tensile strength, lightness units, blueness units and color difference of denim fabrics	Elevated EGII contents increased stone washing effect	Miettinen-Oinonen *et al.*, 1997b, U.S. Pat. 5,874,293
T. reesei, TC*, *Humicola insolens*, EGV, EGV-core *Cellulomonas fimi*, CenA-core	Staining levels, effect of mechanical action	Cellulases without CBDs and mechanical action caused less backstaining	Andreaus *et al.*, 2000
α-amylase, EG V and EG III from e.g. *Scytalidium* (f. *Humicola*), *Fusarium*, *Myceliophthora*, *Trichoderma*	Evaluating streaks and stone-washing appearance	A one-step process for combined desizing and stone-washing	Lund, 1997, WO 97/18286

*TC: total crude cellulase

Genencor has patented *Trichoderma reesei* EGVI (Bower *et al.*, 1998) and they have proposed its use in textile applications. However, no application results have been published thus far. Novo Nordisk (currently Novozymes) has also launched a new commercial bleaching technology, DeniLite™, for denim using laccase (oxidoreductase) enzyme combined with a mediator (Vollmond, 1997). The laccase-mediator system has a specific mode of action in catalyzing the breakdown of the chromophore of color components (Buschle-Diller *et al.*, 2001; Mueller and Shi, 2001). Laccases catalyze the oxidation a variety of aromatic, especially phenolic compounds. Also decolorization of various textile dyes by laccases has been reported (Abadulla *et al.*, 2000). Campos *et al.* (2001a and b) studied the effect of laccases on degradation of insoluble indigo, both in

effluents and on fabrics. According to their results, laccases were able to prevent backstaining of indigo-stained fabrics.

Novo Nordisk has also patented a method in which a stone-washing effect of denim was obtained by using xyloglucan polymer prior to dyeing and afterwards creating the abraded or worn look by enzymatic degradation of the polymer using xyloglucanase (Kalum, 1999). The xyloglucanase is not able to hydrolyse the cellulosic fabric, and therefore no strength loss is resulted.

1.3.2.3 Biofinishing with cellulases

Biofinishing or biopolishing is a term used to describe the removal of surface fuzz (cellulose fibrils and microfibrils) from cellulosic fibres with cellulases. The presence of fibrils leads to fabric problems in wear, notably pilling and a "frosted" appearance, which causes an apparent loss of colour. Softening effects achieved by enzymatic treatment of cotton textiles were already patented by Browning (1974) almost 30 years ago, as reviewed by Heine and Höcker (1995). Enzymatic removal of fibrils results in smoother and cleaner-looking fabrics, which retain their original colour value (Asferg and Videbaek, 1990; Tyndall, 1992). Removal of the fibrils also prevents pilling and subsequently permanently decreases the tendency to form pills during wearing and washing. Other benefits of biofinishing are improved drapeability, fashionable wash-down effects and improved moisture absorbency, the latter being beneficial especially for the home textiles (Clarkson *et al.*, 1992c; Chong and Yip, 1994).

The biopolishing process was patented by Videbaek and Andersen in 1993. Biopolishing/finishing enzymes are suitable both for the wet processes of the textile industry and for finishing textiles either as continuous lengths or as single items in washing machines. The major advantage of enzymatic processes is that they can be adapted to run on already existing equipment in the textile industry. Cellulases are usually applied as multi-component enzyme systems and most commercial cellulases contain a variety of different activities (Nidetzky *et al.*, 1993). Today a commercial mono-component acid endoglucanase cellulase product, Cellulsoft Ultra L (expressed in genetically modified *Aspergillus* fungus) is available from Novozymes (Liu *et al.*, 2000).

Producers of textile enzymes recommend dosages of approximately 0.05 to 6 percent of cellulase preparation on garment weight depending on the desired result, the treatment method and the activity of the enzyme product (Miettinen-Oinonen *et al.*, 1997a). Simultaneously, intensive mechanical contact, generated by the equipment results in breaking off of the fibre ends weakened by enzyme treatment. Due to the unoptimized cellulase composition and high dosages, significant weight and strength losses can occur. A suitable biofinishing effect without excessive loss of fabric strength is generally obtained with 3–5% weight loss of fabric (Lund and Pedersen, 1994).

Several studies and patents have been made to minimize the negative impacts of biofinishing, i.e. weight and strength loss and to better understand the mechanisms of cotton hydrolysis (Table 6). Genencor International, Novo Nordisk A/S and Röhm Enzyme Finland Oy have patented many different fungal cellulase compositions which are either free of or overproduce defined cellulase components (Clarkson *et al.*, 1991, 1992a, c; Miettinen-Oinonen *et al.*, 1997a, b; Lund and Pedersen, 1994). Liu *et al.* (2000) investigated parameters affecting biofinishing effects. They compared the effects of a commercial multi-component acid cellulase, an experimental multi-component endo-enriched cellulase and a commercial mono-component acid endoglucanese cellulase on cotton interlock knitted fabric in laboratory scale. The results indicated that the different cellulases had different selectivities, i.e. the ratios of pilling to fabric burst strength were different, and their sensitivities to liquor ratio and mechanical agitation differed significantly. For optimal biofinishing performance, it is important to select an enzyme product based on cellulase selectivity, existing equipment and fibre type.

In addition to weight and strength losses, dusting can also cause problems especially in the treatment of knitted fabrics (Pere *et al.*, 2001). The fine cotton dust formed in biofinishing processes can cause problems to employees if the dust is not properly removed in an appropriate machine, e.g. in a jet or a tumbler. Repeated inhalation of enzyme-containing dust or mist can also cause respiratory allergies in some individuals (Yingling, 2000; Vanhanen *et al.*, 2000).

Bishop *et al.* (2001) patented a treatment in which the dimensional stability of cotton fabrics was improved by cellulase treatment. These findings were made

using the padding process, different reaction times and various crude *T. reesei* cellulases. After the pad-batch treatments with low levels of selected EG-rich cellulases and standard washing tests they detected good improvement in dimensional stability without high weight and strength losses (Cortez *et al.*, 2002). The reason for the improved dimensional stability is not fully understood although Cortez *et al.* (2002) suggested that partial hydrolysis of fabrics which are not subjected to applied stress during processing could result in stress relaxation in the fibers, yarn and fabrics. Cavaco-Paulo (2001), on the other hand, suggested that the changes in the external broken chains could change the collapsing-twisting behavior of cotton fibres in a drying process.

Biofinishing is also used for the new generation of solvent-spun cellulosic fibres such as Tencel (Acordis Fibers Ltd.) and Lenzing's Lyocell (Kumar *et al.*, 1996; Kumar and Harnden, 1999). The cellulosic fibre lyocell is the first new generic fibre in 30 years. It is defined as a solvent-spun cellulosic fibre prepared from wood pulp in an amine solvent system. Lyocell has high dry and wet tenacity and luxurious hand properties. The unique crystalline structure of lyocell fibres is the basis for a fibrillation. Fibrillation occurs when the fibre is subjected to mechanical action in a wet state and it is most commonly used in the creation of "peach-touch" or "mill-wash" fabrics (Kumar and Harnden, 1999; Taylor, 1998). This process requires three stages: primary fibrillation at a temperature above 90°C with the addition of soda ash to make the fibre swell, cellulase treatment to clean the fabric surface and finally, a secondary fibrillation with high mechanical action. The mechanisms of depilling of lyocell fabrics with engineered *T. reesei* cellulases were studied by Morgado *et al.* (2000) and Kumar and Harnden (1999), who showed that both the removal of microfibrils and their formation can be induced by cellulase enzymes. Kumar and Harnden (1999) also demonstrated that endo-enriched engineered cellulases offer several advantageous features, such as improved hand properties, compared with conventional whole cellulases.

1.3.2.4 Mode of action of cellulases on cotton

There are various different parameters, e.g. synergism of cellulases, pretreatments of cotton, treatment solution conditions, and machinery that affect the ability of cellulases to act on cotton cellulose. The optimum treatment solution conditions for commercial *T. reesei* cellulases are approximately 50°C

and pH 5. The enzyme activity decreases rapidly above 65°C or on both sides of the optimum pH range. The methods of measuring cellulase activities are discussed in section 1.2.1. In order to maintain the optimum pH of the treatment solution throughout processing, an effective buffer system is needed. Cavaco-Paulo *et al.* (1998c) reported that the activities of TC, EG-rich and CBH–rich cellulases on cotton are influenced by ionic strength and adsorbed ionic species. The presence of various metallic ions in the cellulose hydrolysis has been found to decrease cellulase activities (Zeyer *et al.,* 1994).

The effect of dye properties on cellulase hydrolysis of cotton, and vice versa, has been studied using different dye classes, dye molecular sizes, and types of dye/fibre bonding (Traore and Buchle-Diller, 1999; Snyder, 1996; Cavaco-Paulo and Almeida, 1994). The influence of different surfactants on cotton hydrolysis has also been studied intensively, but the results have often been controversial, as reported by Traore and Buchle-Diller (1999).

Due to the large molecular size of cellulases, their accessibility is considered to be limited to large pores in disordered areas and crystallin surfaces. Chenghong *et al.* (2001) investigated the effects of cellulase enzymes on the pore volume and surface area of cotton fabric. Their results showed that the enzymatic hydrolysis decreased the volume and surface area of pores smaller than 60 Å in cotton fabric. The adsorption of cellulases on cellulosic substrates is a prerequisite for subsequent hydrolysis, and cellulase-cotton cellulose binding has been studied by several authors as already described in section 1.3.2.2 (Shen *et al.*, 2002; Azevedo *et al.*, 2000 and 2001; Andreaus *et al.*, 1999, Cavaco-Paulo *et al.*, 1998a; Cortez *et al.*, 2001). The measurements of adsoption are usually based on the determination of free protein concentration in the reaction mixture after incubation of enzyme with substrate.

An overview of research on the action of purified cellulases or cellulases modified by substitution or truncation is presented in Table 6.

Table 6. An overview of patents and research on the treatment of cotton with modified or purified cellulases.

Substrate	Micro-organism	Enzymes	Aim of the study	Properties measured	Ref.
Fibres	*T. reesei*	CBHI, CBHII, EGI	Effects on spinnability	Microscopic analysis, spinnability	Pere *et al.*, 2001
Yarns		CBHI, EGII	Evaluate fabric properties	Yarn evenness, tenacity, hairiness, pilling	Pere *et al.*, 2001
Poplin fabric	*Humicola insolens*	EGV + core	Effect of agitation,	Adsorption/ desorption,	Azevedo *et al.*, 2000
	Cellulomonas fimi	CenA + core	binding	weight loss	
Fabric	*Cellulomonas fimi*	CBD	Binding	Dye affinity, washing fastness, migration, strength loss, reducing ends	Cavaco-Paulo *et al.*, 1999
Fabric	*Trichoderma longibrachiatum*	Cellulase mixture free of CBHI	Provide an improved cellulase composition; decrease strength loss	Strength loss, hand, appearance, colour enhancement, softness, stone-washed appearance	Clarkson *et al.*, 1991, U.S. Pat. 5,246,853
Fabric	*Fungal cellulase*	CBHI-enriched	Provide an improved cellulase composition; decrease strength loss	Strength loss, hand, appearance, color enhancement, softness, stone washed appearance	Clarkson *et al.*, 1992a, WO 93/22428
Fabric	*Humicola insolens*	Mono-component, 43 kD endoglucanace	Improved pilling	Pilling, weight loss	Lund and Pedersen, 1994, WO 96/17994
Fibres, linters	*T. reesei*	CBHI, CBHII, Endo-2	Synergistic action	Reducing sugars, DP, thin-layer chromatography,	Hoshino *et al.*, 1997
	Irpex lacteus	EX-1, En-1		electron microscopy	
	Aspergillus niger	Exo-A, EG-1			
Fabric	*T. reesei*	TC*, CBH-rich, EG –rich	Adsorption	Cellulase activity, adsorption	Cavaco-Paulo *et al.*, 1998c

continues

Table 6. continued

Fabric, Indigo dyed	*T. reesei* *Humicola insolens* *Cellulomonas fimi*	TC* EGV, EGV-core CenA-core	Influence of cellulases on indigo backstaining	Staining levels, effect of mechanical action	Andreaus *et al.*, 2000
Fabric, woven and knitted	*T. reesei*	Over-producing strains (CBH II rich or EG II rich) + purified EGI, EGII, CBHI and CBHII	Provide an improved cellulase composition for treating cellulose-containing textiles	Pilling, weight loss, tensile strength, visual appearance, color	Miettinen-Oinonen *et al.*, 1997a and b, U.S. Pat. 5,858,767 and 5,874,293; Miettinen-Oinonen and Suominen, 2000
Fabric, woven and knitted	*T. reesei*	TC*, EG-rich	Optimizing the use of cellulases in finishing cellulosic fabrics	Pilling, weight loss, tensile strength, drapeability	Kumar *et al.*, 1997
Fabric knitted	*Aspergillus*	TC*, endo-enriched, mono-component acid endoglucanses	Optimization of bio-polishing	Pilling, weight loss, strength loss	Liu *et al.*, 2000
Fabric, woven	*T. reesei*	TC*, CBH-rich, EG-rich	Effects of agitation on the adsorption-desorption behaviour	Weight loss, strength loss, softness, shear and bending hysteresis, bound protein, reducing sugars	Cortez *et al.*, 2001
Fabric, woven and knitted	*T. reesei*	TC*, CBH-rich, EG-rich, genetically modified strains	Improve dimensional stability of fabrics	Weight loss, strength loss, dimensional stability	Cortez *et al.*, 2002; Bishop *et al.*, 2001, WO 01/53592

TC* = total crude cellulase

Table 6 shows that only Pere *et al.* (2001) have performed research with purified *T. reesei* EGs and CBHs on cotton fibers and yarns. Miettinen-Oinonen *et al.* (1997b, U.S. Pat 5,874,293) patented the finding that improved stonewashing properties could be obtained by adding purified *T. reesei* EGII cellulase to the complete cellulase composition. Mono-component commercial *Aspergillus* cellulase was used by Liu *et al.* (2000) in the evaluation of biofinishing.

However, detailed information concerning the cellulases was not given, and dosages of the cellulases compared were reported as % on the weight of the fabric. The other studies listed were performed with enzymes derived from modified bacterial or fungal organisms.

1.3.3 Enzymes in processing of bast fibres

Bast fibres such as flax, hemp, jute and kenaf are composed of cellulose (over 50%), hemicelluloses, lignin, pectins, fats and waxes. Of the bast fibres, flax is the oldest and most widely used plant, supplying both oil seed and fibres to produce linen for textiles (Akin *et al.*, 2000a). The bast fibres of flax, which are situated in the stem between the cortex and the hollow central wood, cannot easily be separated from the other plant tissues unless some decomposition of the stem takes place. This process of decomposition is called retting. Traditionally, two types of retting have predominated, namely dew- and water retting, which rely on indigenous soil fungi or on bacteria in an aqueous environment, respectively. Chemical retting, i.e. use of acid, base, surfactants and chelators, has also been tested for the separation of fibres from non-fibrous tissues (Evans *et al.*, 2002a). Retting has always been one of the major costs and practical limitations to the more widespread use of flax. Various attempts have been made since the late 1970s to introduce more rapid and controllable enzyme retting processes to replace the conventional dew- and water-retting processes (Sharma and van Sumere, 1992; Akin *et al.*, 1997, 2000a, b). Several different enzymes, e.g. pectinases, cellulases and hemicellulases have been tested (Evans *et al.*, 2002a, b; Akin *et al.*, 1997). In addition to enzyme evaluation different methods such as tank and spray retting have also been studied (Akin *et al.*, 2000b). Studies on the use of enzymatically retted flax for spinning on common cotton machinery in blends with cotton were reported by Kimmel *et al.* (2001). Development of such methods requires knowledge of the chemical and physical characteristics of flax (Gamble *et al.*, 2000; Akin *et al.*, 1997; Buchert *et al.*, 2001). Despite the progress made during this period, no enzymatic retting method has hitherto been commercialized (Akin *et al.*, 2000b).

Cellulase finishing is also increasingly being investigated on other cellulosic fibres such as bast fibres and viscose as well as on cotton and lyocell. The different organization and crystallinity of cellulose in different fibres leads to

differences in cellulase adsorption and activity (Shen *et al.*, 2002). Buschle-Diller *et al.* (1994) showed using *Trichoderma viride* cellulase that removal of surface fibrils from linen and ramie fabrics can be accomplished without high weight losses or reduction in tensile strength. Pere *et al.* (2000) tested the use of purified *T. reesei* CBHI and EGII cellulases on linen fabric. They reported that the EGII treatment had positive effects, i.e. improved pilling properties on linen. On the other hand, linen fabric appeared to be especially vulnerable to strength losses caused by endoglucanases.

1.4 Aims of the study

The aim of this work was to define the optimum cellulase compositions for denim washing and biofinishing of different types of cotton fabrics in order to achieve the desired finishing effects with minimum negative impacts such as strength and weight losses. The specific topics of the study were:

- To investigate the individual effects and mechanisms of hydrolysis of cotton cellulose by purified cellulases

- To study the synergistic effects between different cellulases on cotton fabrics

- To verify the results obtained with purified cellulases by using experimental cellulase mixtures.

2. Materials and methods

2.1 Cotton substrates

All substrates used in this study were 100% cotton and they are listed in Table 7.

Table 7. Cotton substrates used for cellulase treatments.

Type		Pretreatments	Weight g/m^2	Picks and ends /cm	Ref
KNITTED	Interlock	Scoured, peroxide bleached	203	-	(II, IV)
WOVEN	Twill	Scoured, bleached	250	30, 21	(I)
	Twill, open-end spun	Scoured, peroxide bleached	223	26, 18	(IV)
	Twill, ring-spun	Scoured, peroxide bleached	237	27, 17	(IV)
	Poplin woven	Scoured, bleached	100	60, 32	(I, V)
	Denim	Desized	473	15, 28	(III)

2.2 Enzymes

Endoglucanase I, II (EGI, EGII) and cellobiohydrolase I, II (CBHI, CBHII) were purified from a *Trichoderma reesei* culture filtrate by chromatographic methods as described previously by Pere *et al.* (1995) and Rahkamo *et al.* (1996). Protein concentrations of the purified enzyme preparations were assayed according to Lowry *et al.* (1951) after precipitation with trichloroacetic acid. The endoglucanase activity, listed in Table 8, was measured using hydroxyethylcellulose as substrate (Bailey and Nevalainen, 1981). Molecular weights and pI values of purified cellulases are presented in Table 3.

Table 8. Specific endoglucanase activities (nkat/mg), and protein contents of purified (a) and commercial (b) cellulases. One nkat is defined as the amount of enzyme producing one nanomole of reducing sugars as glucose in one second.

a)

Enzyme	Specific endoglucanase activity nkat/mg	Protein content (mg/ml)
CBHI	Not detectable	25
CBHII	Not detectable	3.9
EGI	540	2.98
EGII	1170	1.67

b)

Enzyme	Specific endoglucanase activity nkat/mg	Protein content (mg/ml)
Biotouch L	70	195
Ecostone L	160	100
Cellulase B	8	110

Two different commercial whole acid-type cellulase mixtures derived from *Trichoderma reesei* (Ecostone L and Biotouch L) were kindly provided by AB Enzymes, formerly Röhm Enzyme Finland Oy. Ecostone L used in stone washing and Biotouch L used in biofinishing of cellulose-based fibres both contain endo- and exocellulases. In addition to cellulases, commercial cellulase mixtures may also contain minor amounts of other enzymes. Cellulase B used in denim treatments (III) is a CBH-rich experimental cellulase preparation produced by a genetically modified *Trichoderma reesei* strain. Cellulase B did not contain endoglucanases I or II and the amount of cellobiohydrolases I and II had been increased in comparison to the original whole acid cellulase preparation. The experimental cellulase mixtures A–F with different cellulase

profiles were produced by genetically modified *T. reesei* strains (Table 9). The strains were constructed using standard genetic engineering techniques and as described previously by Suominen *et al.* (1993) and Miettinen-Oinonen and Suominen (2002) (IV).

Table 9. Different Trichoderma reesei cellulase samples.

Code	Cellulase overproduced	Gene deleted	Main cellulases present (in percentage)
A		*egl2, cbh2*	CBHI (85), EGI (15)
B		*egl1, cbh2*	CBHI (75), EGII (25)
C		*Egl1, cbh1*	CBHII (75), EGI (25)
D		*Egl1, cbh1*	CBHII (75), EGII (25)
E	EGII	*cbh1*	EGI (11), EGII (84), CBHII (5)
F	EGII		EGI (10), EGII (20), CBHI (50), CBHII (20)

2.3 Enzyme treatments

The enzymatic treatments were typically carried out in a Linitest machine (Atlas, USA) for one (I, III, IV), two (II) or 4 hours (V) at 50°C using 2 to 10 g of cotton swatches in stainless 500 ml pots. The mechanical action was provided by adding five or ten steel discs (mass of each disc 20 g ± 2 g) to the pots (I, III, V). The other conditions in the treatments were: 0.05 M citrate or acetate (V) buffer, liquor ratio of 1:25 (V), 1:20 (III) or 1:10 (I, II, IV, V), pH 5.0. All the cellulases were dosed on the basis of protein amount in the enzymatic treatments. Dosages of the purified, experimental and commercial cellulases were 0.01–10.0 mg/g. When used in combinations the protein ratios between CBHs and EGs were 0/100, 25/75, 50/50, 75/25 and 100/0% (II). In addition, combinations of EGs and CBHI of 5/95, 15/85, 25/75 and 35/65% were used (unpublished data). Non-ionic surfactant Berol, 0.1% of fabric weight (Berol Nobel AB, Sweden), was added to denim treatments (III).

After the treatment the pots were immersed in boiling water for 5 minutes in order to inactivate the enzymes activity, whereafter the fabric samples were rinsed with hot and cold tap water and air dried. Reference treatments were carried out in similar conditions without enzyme addition. Each treatment was carried out in duplicate or four times.

2.4 Analysis

The analyses carried out during this study are listed in Table 10.

Table 10. Analyses used in cotton treatments.

Analysis	**Method used**	**Ref.**
Reducing sugars	DNS	Bernfeld, 1955
Solubilized cellooligosaccharides	HPLC (Dionex)	Tenkanen *et al.*, 1997
Protein	Lowry	Lowry *et al.*, 1951
Molecular-weight distribution	GPC, after dissolution in LiCl DMAC	Westermark and Gustafsson, 1994.
Bursting strength	Psi-burst, GWB	(ISO 2960)
Tear strength	Alwetron TCT 10	(SFS 3981)
Weight loss	Dry weight, conditioning for 24 h, 20°C, 65% RH	
Pilling	Martindale Atlas	(ISO 12947-1) (ASTM D 3512-82, D 1375-72)
Bending, shear	Kawabata	
Wrinkle recovery	Monsanto method	(ATM D 1295)
Microscopy	SEM	
Color	Minolta Chroma meter (CIELab system) Spectrophotometer (absorbance at 619 nm)	

2.4.1 Filtrate analyses

Reducing sugars were measured in the treatment filtrates by the dinitrosalisylic acid method (DNS) using glucose as standard (Bernfeld, 1955) (I, II, III, V). The solubilized oligosaccharides liberated in the enzyme treatments were analyzed by HPLC (high performance liquid chromatography) (Tenkanen *et al.*, 1997) (II, V). The amount of indigo in the treatment solution was measured as absorbance at 619 nm against buffer solution using a spectrophotometer (Mark and Tsim, 1994) after a secondary enzymatic hydrolysis of the solubilized cellulose oligosaccharides to monomers (Buchert *et al.*, 1993) (III). The colour of the treatment solution was also monitored by measuring lightness as reflectance units (L-value) with a Minolta Chroma Meter spectrophotometer (CIELab system, Minolta Camera Co., Osaka, Japan). For this analysis 25 ml of the treatment solution was placed in a Petri dish and five colour measurements were carried out from each sample (III).

Cotton powder produced during cellulase treatments of cotton poplin fabric was obtained after sedimentation and centrifugation from the treatment baths. Molecular weight (MW) parameters of number-average MW ($M_n = (\Sigma M_i N_i)/\Sigma N_i$), weight-average MW ($Mw = (\Sigma MiWi)/\Sigma W_i$), and polydispersity ($P_d$, the ratio of M_w/M_n) were determined by gel permeation chromatography (V).

2.4.2 Fabric analyses

Fabric weight loss was measured after drying for 4 hours at 105°C with subsequent overnight cooling in a desiccator (II, IV) or after conditioning for 24 h at 20°C and 65% RH (I, V). Bursting strength of treated knitted fabrics was measured with a Psi-Burst tester according to ISO standard 2960 (II, IV).The tear strength of treated woven fabrics was measured on an Alwetron TCT 10 machine with the 100 N cell (SFS 3981). Tear strength was measured in the warp and weft direction in poplin samples and only in the warp direction in the twill samples (I).

The effects of the cellulases on pilling tendency was measured by the ATLAS Random Tumble Pilling Tester according to the standard ASTM D 3512-82 (II,

IV) or by using the modified Martindale abrasion testing method, with 500 or 2000 cycles of abrasion (I, IV).

Wrinkle recovery of the fabrics was tested by the Monsanto method (ASTM D 1295) along the warp direction (I). Fabric bending hysteresis was measured using a KES-FB2 instrument from Kato Tech Co. Ltd at 20°C and 65% RH (I).

The effects of the cellulase treatments on fabric colour were monitored on the right side of the denim swatches by measuring the L-value with a Minolta Chroma Meter using the CIELab system (III). The stone washing effect was also evaluated by a panel of five randomly selected persons. Each fabric was rated once on a score from 1 to 5 (5 being the best result with good stone washing effect, 1 being the rating for the reference fabric) (III).

Scanning Electronic Microscopy photographs (Leica Cambridge Stereoscan 360) were obtained after 2 minutes of gold metallization (Bio-rad SC 502) of the fabric samples (unpublished data).

3. Results and discussion

3.1 Treatment of cotton fabrics with monocomponent cellulases

3.1.1 Cellulase treatment of woven fabrics (I, V)

Two different types of cotton fabrics, i.e. cotton twill (250 g/m^2) and cotton poplin (100 g/m^2) were treated with purified *Trichoderma reesei* cellulases CBHI, EGI and EGII (I) and in addition with CBHII (V) in order to evaluate the effects of the individual cellulases and to obtain more information on the role of fabric type on the effects of enzyme treatment. The dosages of purified cellulases were 0.2–5.0 (I) and 10 (V) mg of protein per gram of fabric and the treatment time was 60 minutes (I) or four hours (V).

In this part of the study, additional mechanical agitation was achieved by adding ten (I) or 5 (V) stainless steel discs to each Linitest pot. Zeyer *et al.* (1994) and Cavaco-Paulo *et al.* (1996a) have pointed out the importance of mechanical agitation during enzymatic hydrolysis and how it plays a role in opening up the substrate and allowing the enzyme to cleave the cellulose chain. Cavaco-Paulo *et al.* (1996a) also reported that the effect of the endocellulases in cellulase mixtures increases with mechanical agitation, whereas the relative exoactivity is reduced. Kumar *et al.* (1997) and Liu *et al.* (2000) listed the factors that affecting mechanical action, *i.e.* equipment type and design, process time, load size, liquor ratio and rotation speed.

The impact of monocomponent *Trichoderma reesei* cellulases on the weight loss of twill and poplin fabrics was tested (I). EGII was found to be most efficient in the hydrolysis cotton cellulose with an enzyme dosage of 5 mg/g, causing 2.3% and 2.4% weight losses of twill and poplin fabrics, respectively (Figs. 1a and 4a/I). Only a slight increase in twill weight loss was obtained by increasing the EGI and CBHI dosage from 0.2 to 5 mg/g. When the 2.5-folds lighter poplin fabric was used EGI and CBHI treatments resulted in significantly higher weight losses (Figs. 1a and 4a/I). Reference treatments showed that the mechanical action used in the Linitest caused higher weight loss of poplin than of twill fabric.

When the longer treatment time (four hours) and higher enzyme dosage (10 mg/g) were used on poplin fabric, again the highest weight loss was obtained with EGII (3.1%), compared to the weight losses caused by EGI, CBHI and CBHII, i.e. 1.9%, 1.5% and 0%, respectively (Figure 1A/V). By adding five stainless steel discs in the treatment solution, major increases in weight losses caused by EGII and EGI were observed, i.e. 51.8% and 22.1%, respectively. This result supports the findings of Cavaco-Paulo *et al.* (1996a) and Azevedo *et al.* (2000) reported above. Of the endoglucanases, it was clearly shown that the mechanical action had a greater effect on EGII than on EGI, measured as weight loss of the fabric. Mechanical action had a less pronounced effect on CBHI and CBHII treatments.

The effect of enzyme treatments on the strength properties of twill and poplin fabrics was measured (I). CBHI treatment had practically no effect on the strength properties of the twill fabric, even at the highest enzyme dosage of 5 mg/g (Figure 1 b/I). When the fabrics were treated with endoglucanases at dosages of less than 1 mg/g, only slight effects on tear strength were observed. However, by increasing the EGII dosage to 5 mg/g, significant reduction in strength properties of twill and poplin was obtained. EGI, on the other hand, did not cause any further strength loss at increased enzyme dosages (Figs. 1b and 4 b/I).

When strength loss was plotted against weight loss, it was observed that at low weight loss levels of twill fabric the strength loss caused by EGI was more pronounced. In the case of poplin at a weight loss level of 1%, about 10% strength loss was obtained with all the enzymes tested (Figs. 1c ad 4c/I).

In general poplin fabrics have more interlacing points than twill fabrics. Therefore, the propensity of poplin fabric to pilling was less pronounced than that of twill fabric. In the evaluation, the panel value of the control was set to unity in order to facilitate comparison of the enzymes. The drawback of this method was that the panel values of poplin were not comparable with those obtained on twill. Endoglucanases were capable of improving pilling abrasion resistance of twill and poplin fabric even at the lowest enzyme dosage of 0.2 mg/g. Of the endoglucanases, EGII had the more pronounced effect when twill fabric was treated and the panel value increased from 1.2 to 4.8 at the 5 mg/g dosage level (Fig 3a and b/I). However, in the case of poplin, a less pronounced

effect of pilling resistance of EGII at the dosage of 5 mg/g was observed, possibly due to high degree of hydrolysis of the fabric. The EGII attack and mechanical agitation may have caused more protruding fibres on the fabric surface by cutting the cellulose chains. Simultaneously, high strength loss (about 40%) and weight loss (2.4%) of the poplin fabric were observed. In practice such high strength loss values are not acceptable. In summary, the endoglucanases had a positive effect on pilling at relatively low enzyme dosages, in which practically no strength decrease was observed.

The enzymatic treatments caused no significant changes in wrinkle recovery values of the cotton twill or poplin fabrics according to the Monsanto measurements. In the case of twill, EGI and EGII treatments increased the wrinkle recovery degree slightly from 53 to 62 and 60, respectively (Table I/I).

All purified enzymes had a slight positive effect on the drapability of twill and poplin when used at low dosages, as measured by bending hysteresis (2HB) (I). By increasing EGI dosage in the twill treatment an improvement in 2HB was observed, whereas with poplin the effect of EGI was less pronounced (Figs. 2a and b/I). However, no effect was obtained by increasing the dosages of CBHI and EGII, which could be due to increased friction between the fibres. These results are in agreement with the results obtained by Cortez *et al.* (2001). They treated cotton twill fabric with *T. reesei* total crude (TC) cellulase in real textile processing machinery, i.e. in jet and winch machines with different enzyme dosages. Their results showed that the measured effects of cellulase treatments, i.e. weight and tear strength loss, amount of reducing sugars liberated, reduction in shear and bending hysteresis and softness, were always greater in the jet, but that the differences between jet and winch were not directly related to cellulase concentration, i.e. smaller enzyme dosages in jet treatments reduced the bending hysteresis values more than higher dosages.

A combination of enzyme action and high level of agitation may generate a stubble of fibrillar material on the fibre surface, as already discussed and reported by Cavaco-Paulo *et al.* (1996a). In their study they used the same poplin fabric treated with a total *T. reesei* cellulase TC (Cellusoft L) and/or an experimental sample of EG (with unclearified purity) and compared the effect of mechanical agitation on bending hysteresis (2HB) in laboratory scale. In general, low 2HB values (low interfibre friction) indicate the softness (Postle, 1989;

Bishop, 1996) and drape (Collier and Epps, 1999) of the fabric. In the study of Cavaco-Paulo *et al.* (1996a), the mechanical action was achieved by twelve steel discs in Linitest pots. The authors used a constant enzyme dosage of 1 g/l (the amount of protein was not reported) and increasing treatment times from 20 to 120 min. Their results showed that low-agitation EG treatment produced little change in fabric bending hysteresis, whereas high-agitation EG treatment caused a progressive increase in 2HB. Treatments with TC in both low and high agitation experiments caused reduction in 2HB. They assumed that EG treatments with high levels of mechanical agitation make fabrics feel harsher, whereas all TC treatments may provide fabric-softening benefits.

By comparing the different fabric properties at the same weight loss level, it was apparent that CBHI and EGs have different effects on twill and poplin fabrics (I). This study showed that EGs caused more strength loss than CBHI but had a more positive effect on bending properties and pilling. However, at low hydrolysis levels practically no strength loss was obtained with the EGs, whereas at the higher dosages EGII caused significantly higher strength losses as compared to EGI. Subsequently, by optimising the endoglucanase dosage, a positive effect on pilling tendency could be obtained without any significant reduction in fabric strength properties.

3.1.2 Cellulase treatment of knitted fabrics (II, unpublished data)

To evaluate further the effects of monocomponent *T. reesei* cellulases on different fabric types, cotton knitted interlock fabric was treated with CBHI, CBHII, EGI and EGII. In this study treatments were carried out using a total enzyme dosage of 5 mg protein/g cotton (II). No additional mechanical action was used in the Linitest treatments.

The degree of hydrolysis of fabric was monitored by measuring the amount of reducing sugars solubilized by the dinitrosalicylic acid method with glucose as standard (Table 1/II). The different modes of action of exo- and endoenzymes could be visualized in the oligosaccharides solubilized from the fabric and analysed by HPLC (Fig. 1/II). The predominant hydrolysis product of CBHI and CBHII was cellobiose, whereas EGI and EGII also solubilized larger oligosaccharides. When the weight losses were compared with the reducing

sugar analysis, it was observed that the reducing sugar analysis resulted in clearly lower values, indicating the formation of higher DP hydrolysis products and cotton powder. EGI resulted in the highest weight loss (1.3%), followed by CBHI (1.0%).

EGII also caused the highest strength loss (7.8%) in these tests, whereas the other cellulases had practically no effect on the strength properties (Table I/II). CBHI and CBHII did not affect the pilling properties of the interlock knit, whereas EGI and EGII treatments improved the pilling values significantly (Table I/II). Similar results were obtained earlier with twill and poplin fabrics (I), as discussed previously in section 3.1.1.

The hydrolysis levels obtained in interlock treatments were clearly lower compared to previous work with twill and poplin fabrics as substrates (I). Furthermore, some differences in the strength losses with endo- and exoenzymes were observed. As previously discussed in section 3.1.1, the different results were probably caused by the use of mechanical action in woven fabric treatments.

The results presented earlier in Table I/II and in section 3.1.1 show that purified EGII alone had a positive effect on pilling properties. In order to further elucidate the effect of EGII on the properties of cotton interlock, smaller enzyme dosages (0.01–5 mg/g) of EGII and Biotouch L (a cellulase mixture produced by *T. reesei*) and shorter treatment times, i.e. one hour were used (unpublished data). The results are shown in Table 11. In the one hour treatment even the smallest dosage, i.e., 0.01 mg/g of EGII was sufficient to improve the pilling properties of knitted fabric from 2–3 to 3–4. Small EGII dosages, based on the total protein amount, also resulted in significantly lower strength loss as compared with the same Biotouch L dosages. Percentage weight loss and the amount of reducing sugars liberated from EGII-treated fabric were also negligible, indicating low dust formation. These results are analogous to those obtained by Liu *et al.* (2000) with *Aspergillus*-derived mono- and multi-component cellulases. These authors demonstrated the greater selectivity of mono-component endoglucanase cellulase for hydrolyzing surface fibres with very limited fabric strength loss compared to endo-enriched and TC acid cellulases. It should be noted that commercial cellulase mixtures, e.g. Biotouch

L, may also contain minor amounts of other enzymes, such as hemicellulases, thus decreasing the true dosage of cellulases.

Table 11. Effects of different dosages of EGII and Biotouch L on cotton interlock properties (Unpublished data).

Sample	Dosage mg/g	Weight loss %	DNS % of d.w.	Strength loss %	Pilling value
EGII	5	1.89	0.27	13.7	5
	2.5	1.44	0.20	5.4	5
	1	0.91	0.18	8.1	4–5
	0.5	0.63	0.14	9.3	4–5
	0.25	0.51	0.10	4.6	4
	0.1	0.38	0.07	2.4	4
	0.01	0.39	0.04	1.8	3–4
Biotouch L	5	2.91	1.53	8.9	5
	1.0	1.1	0.71	8.2	4
	0.5	0.85	0.47	5.5	4
	0.25	0.61	0.28	4.0	3–4
	0.1	0.55	0.19	4.3	3–4
	0.01	0.35	0.05	3.7	2–3
Ref.	-	0.18	0.04	-	2–3

3.1.3 Cellulase treatment of denim (III)

The effects of purified cellulases, EGI, EGII and CBHI on denim washing were also evaluated and compared to the effects obtained with two different cellulase mixtures. The cellulase mixtures Ecostone L and Cellulase B were derived from *Trichoderma reesei*. Ecostone L was a commercial whole acid cellulase preparation used in stone washing and contained both endo- and exoactivities. Cellulase B was a CBH-rich experimental cellulase preparation produced by a genetically modified *T. reesei* strain. Cellulase B did not contain endoglucanases

I or II, and the amounts of cellobiohydrolases I and II were increased in comparison to the original whole acid cellulase preparation. The specific endoglucanase activities present in the enzyme preparations are listed in Table I / III.

Stone washing effects were evaluated by analyzing the hydrolysis of denim by measuring the formation of soluble reducing sugars from cotton fabric. Of the purified cellulases EGI caused a slightly higher hydrolysis degree with all the enzyme dosages measured. EGII and CBHI were almost unable to solubilize indigo-dyed denim cellulose even at the highest dosages (Figure 1 / III). When the enzyme dosages were low, no differences in the hydrolysis efficiency between purified cellulases and the cellulase mixtures were observed. However, with the highest enzyme dosages, cellulase mixtures released significantly higher amounts of reducing sugars. The high amount of reducing sugars liberated in Cellulase B treatments could have been an artefact due to the high amount of CBHs in the preparation, leading to production of cellobiose from the already solubilized oligosaccharides. Thus the amount of reducing sugars increased although no further solubilization of cotton occurred. Similar levels of reducing sugars were obtained (Table 11) when undyed knitted cotton was treated with Biotouch L and EGII.

The treatment solution of denim hydrolysis was also monitored by measuring the dissolved color as absorbance of the filtrates after secondary enzymatic hydrolysis and by measuring lightness as reflectance units. Despite the very low hydrolysis level, the cellulase most efficient at dissolving indigo was EG II with all enzyme dosages (Figure 2. A and B / III).

The properties of denim swatches were also evaluated by reflectance units and by a panel. These measurements showed that EGII was the most efficient cellulase at removing color from denim, producing a good stone washing effect with soft fabric hand (Table II / III). On the other hand, CBHI had no stone washing effect, even at high enzyme dosages (Table II / III).

These results are analogous to the results patented by Miettinen-Oinonen *et al.* (1997b), showing that an increased level of EGII in the cellulase composition of *T. reesei* imparts an acceptable stone-washed appearance to denim. Clarkson *et al.* (1992b) patented the finding that fungal cellulases free of CBHs yield better

stonewashing properties with less backstaining. In their patent, the cellulase mixture contains at least about 40 weight percent of endoglucanase III derived from *Trichoderma sp.*

3.1.4 Effects of enzymatic treatments on the molecular weight distribution of cotton powder (V)

Measurements of molecular weight and molecular weight distribution of cellulose before and after cellulase treatment provide the possibility to evaluate and optimize the effects of enzymatic hydrolysis on a fundamental property, i.e. the length of the cellulose chain (Rousselle and Howley, 1998). The chain length, or degree of polymerisation (DP), of a cotton cellulose molecule may be as high as 14 000, but it can be easily reduced to 1000–2000 by different purification treatments with alkali (Nevell, 1995). According to Battista (1950), the crystalline regions have a DP of 200–300. Correspondingly, the molecular weight (MW) of cotton usually lies in the range of 50 000–1 500 000 (Sundquist, 1983).

The type and molecular weight distribution of the hydrolysis products obtained after treatment of cotton poplin with EGI, EGII, CBHI and CBHII cellulases with a dosage of 10 mg/g were studied. When high mechanical action was used in the cellulase treatment, EGII was the only enzyme which reduced the molecular weight of cotton powder (Table 2/V). Furthermore, EGII caused higher weight loss of a fabric than EGI, i.e. 52% and 22% respectively. Azevedo *et al.* (2001) showed in their study that an EG-rich *T. reesei* preparation reduced the DP of the cotton powder, derived from the same cotton poplin, more than TC and CBH-rich preparation after long hydrolysis (24 h) and high enzyme dosage when mechanical action was involved. These results support the findings obtained in this part of the study.

When low mechanical action was involved, all cellulase treatments reduced the weight-average MW of cotton poplin powder, and the peaks of the MW distribution curves were shifted to a lower molecular weight compared to the powder produced in reference treatment. The number-average MW was slightly increased after all cellulase treatments. Polydispersity was decreased by all cellulases, indicating narrower distribution curves (Table 2 and Figs. 3 and 4/V).

The degree of hydrolysis was also monitored by measuring the amount of solubilized reducing sugars by the DNS method and by HPLC by measuring the solubilized oligosaccharides liberated. The different modes of action of the exo- and endoenzymes were observed: all the cellulases liberated cellobiose, whereas only EGII also liberated cellotriose (Table 1/V). Higher mechanical action increased the amount of solubilized oligosaccharides, especially when CBHI, EGI and EGII were used (Table 1/V).

Kleman-Leyer *et al.* (1994) reported that *T. reesei* CBHII was not able to affect the molecular size of cotton cellulose. However, EGI was able to decrease the DP of cotton cellulose during prolonged incubation (192 hours). Their size exclusion chromatography measurements showed a single peak, which shifted with time to progressively lower degrees of polymerization. In the later stage of hydrolysis, when a weight loss of 34% was observed, this peak was centered over a DP of 200 to 300, the postulated size of the crystalline regions. These results support well the finding that EGI has relatively low activity towards crystalline cellulose (Srisodsuk, 1994). Rousselle *et al.* (1998), on the other hand, did not observe any reduction in MW of cotton cellulose after three hours of total cellulase (Cellusoft L, *Trichoderma* origin) treatment in a Launderometer despite the high weight loss (20%) and breaking load reduction (77%) of cotton printcloth fabric.

The results obtained in this part of the work (V) showed that EGII treatment with high mechanical action is able to reduce the molecular weight of cotton powder more than EGI treatment. It was also seen that EGII caused much higher weight loss in cotton fabric when mechanical action was involved. This could be due to the different activities of EGs: EGII shows higher activity towards crystalline celluloses than EGI (Cortez, 1999).

3.1.5 SEM analysis of cellulase-treated fabrics (unpublished data)

The effects of the monocomponent cellulases CBHI, EGI and EGII, combined with mechanical action, on cotton twill and interlock fabrics were examined by microscopy. Twill treatments were carried out according to the methods described in paper I with an enzyme concentration of 5 mg protein per gram of fabric. Scanning Electronic Microscopy photographs (Leica Cambridge

Stereoscan 360) were obtained after 2 minutes of gold metallization (Bio-Rad SC 502) of the fabric samples.

After EGII treatment with mechanical action, a clear increase of cracks and fibrils on twill fibers was observed compared to reference, CBHI and EGI treatments (Figure 5). These photos support our previous findings (paper V) that the mechanical action has a greater influence on EGII treatments than on treatment with the other tested cellulases. It was also reported by Cavaco-Paulo and Almeida (1994) that the microfibrils formed during EG-rich cellulase treatments can increase pilling in controlled conditions. However, despite high fibril and crack formation of enzyme treated fibers, the pilling results presented in paper I show an excellent pilling value (4.5–5) of EGII-treated twill fabric. One explanation for this could be that the fibrils formed in EGII treatment are so weak that they are cut off by the mechanical action of the pilling tester (ASTM D 3512-82). Unfortunately, weight loss values caused by tumbling in the pilling tester were not measured.

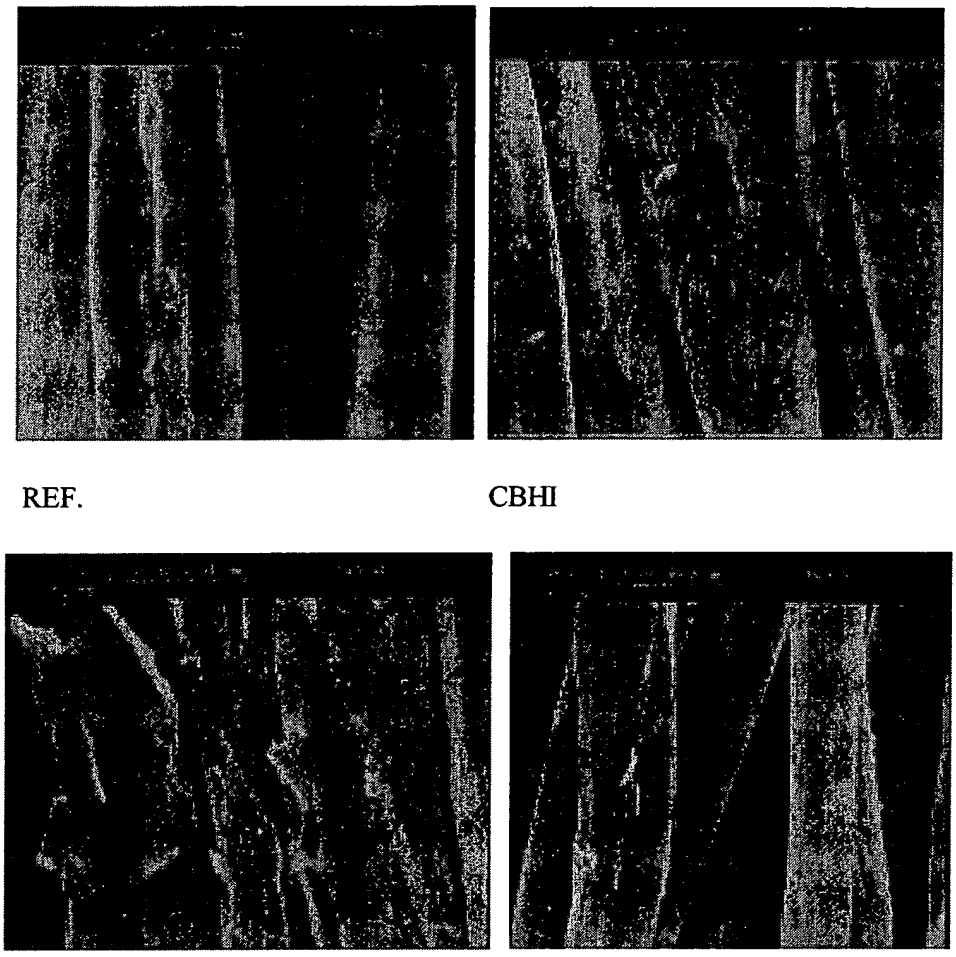


Figure 5. Scanning electron micrographs of cotton twill fabric treated with CBHI, EGI and EGII (5 mg/g, 1 h, + additional mechanical agitation). Reference treatment without enzymes is also shown.

To verify the finding above and to study the possible correlation between surface polishing effect and pilling formation, SEM photos were taken after 2 hour EGII, CBHI and reference treatments before and after pilling testing ASTM D 3512-82. The knitted fabric and treatment conditions used are described in paper II. The pilling values of treated fabrics are also shown in paper II in Table I. Although the reference treatment and CBHI treatment had very low pilling values, i.e. 1, and a large number of pills were observed on the fabric surface, no clear difference could be seen in the SEM photos. Lower fibril and crack formation of EGII-treated fibres could be partly explained by lower mechanical action, i.e. no additional steel discs were added to the Linitest pots.

These findings show that high pilling propensity of fabric could not be visualized by SEM photos.

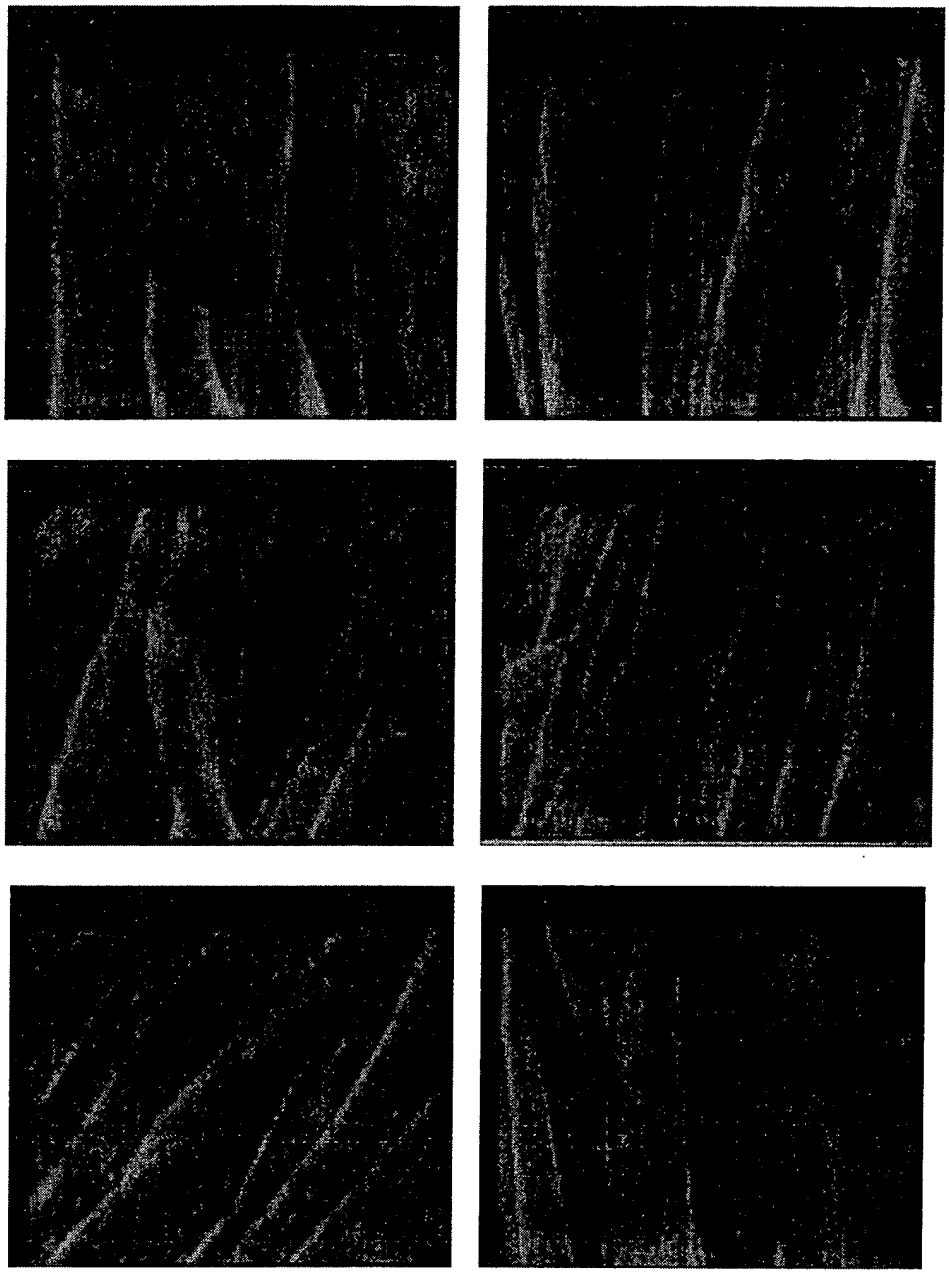


Figure 6. Scanning electron micrographs of CBHI- and EGII-treated cotton knitted interlock fabric and reference treatment before and after pilling test (ASTM D 3512-82).

Several microscopical studies have been made to evaluate and visualize the effects of cellulases on cotton fibers (Cavaco-Paulo, 2001; Cavaco-Paulo *et al.*, 1994 and 1996b; Buschle-Diller *et al.*, 1994). Cavaco-Paulo *et al.* (1994 and 1996b) showed in their SEM photos a clear increase of microfibril formation when mechanical action was included in the cellulase treatments.

3.2 Synergism of *Trichoderma reesei* cellulases in finishing of cotton (II, IV)

Cotton knitted interlock fabric was treated with different *T. reesei* cellulase combinations, i.e. endo-endo, endo-exo, and exo-exo in order to evaluate the synergistic effects of these enzymes. The treatments were carried out using a total enzyme dosage of 5 mg protein/g cotton. The ratios between EGs and CBHs were 0/100, 25/75, 50/50, 75/25 and 100/0%. No additional mechanical action was used in the Linitest treatments. The synergistic effects of different cellulases were monitored by weight loss and reducing sugar analysis. According to weight loss analysis, both endoglucanases exhibited clear synergism with CBHI. EGI also showed slight synergism with CBHII (Fig. 2a and b/II). Of the endo-exo synergisms, the EGI/CBHI synergism was most pronounced and ratio of 25/75 of EGI/CBHI caused a weight loss of 2.1%. CBHI/CBHII mixtures caused no significant synergistic effect in weight loss. The treatment with EGI/EGII mixtures, by contrasts, decreased the weight loss compared to weight losses caused by either of the EGs alone (Fig. 2c/II). The synergism between different purified cellulases as measured by weight loss is summarised in Table II/II. By comparison, treatment with commercial Biotouch L (protein dosage 5 mg/g) containing all cellulases resulted in weight losses of 1.8%. Thus, tailored EGI/CBHI mixtures caused higher weight loss compared to the Biotouch L treatment.

The synergism between EGs and CBHI was also observed in the reducing sugar analysis of the reaction filtrates. The use of 25, 50 and 75% of EGI in the mixture with CBHI increased the hydrolysis level from 0.4% up to 1.1, 1.2, and 1.0% (dry weight), respectively (Figs. 3a and b/II). EGI/CBHII synergism had a less pronounced effect on the hydrolysis. The lower hydrolysis levels obtained with mixtures of EGII with CBHI or CBHII as compared to EGI in similar combinations could also be visualized (Figs. 3a and b/II). When EGI and EGII

were combined, there was no increase in the amount of solubilized sugars. This indicates that in the hydrolysis of cotton interlock, the two endoglucanases act at the same sites of the substrate and therefore do not exhibit synergism. Rahkamo *et al.* (1998) reported similar results with dissolving pulps. Table 12 shows the synergism between *T. reesei* cellulases as measured by reducing sugars.

Table 12. Synergism between T. reesei cellulases on cotton interlock measured by liberation of reducing sugars. Symbols: + indicates synergism, - indicates no synergism.

	CBHI	CBHII	EGI	EGII
CBHI		+	+++	++
CBHII	+		+	+
EGI	+++	+		-
EGII	++	+	-	

Despite the high weight loss obtained with the combination EGI/CBHI in all mixture ratios, practically no reduction in strength properties was observed. On the other hand, almost all combinations including EGII present resulted in higher strength losses despite low weight losses, the only exception being the EG II/CBH I combination with a ratio of 25:75 (Fig 4/II).

When the pilling tendencies of the cellulase-treated fabrics were analyzed, it was observed that neither high weight loss nor strength loss corresponded to improved pilling properties. Similar observations were made by Liu *et al* (2000) with *Aspergillus* commercial mono-component cellulase. In all cases, EGII–based combinations resulted in good pilling properties (Table III/II). Compared to the treatment with Biotouch L with a panel score value of 4.5, the EGII/CBHI combinations were almost equally effective, having scores of 4 in all ratios. The EGII/CBHI ratio of 25/75 caused almost no decrease in strength despite the high improvement in pilling resistance. On the other hand, EGI combinations, despite higher weight losses, were slightly less efficient in improving pilling resistance of cotton interlock compared with EGII-based mixtures. Compared to cellulase mixtures, EGII treatment alone resulted in a pilling level of 4 with very low

weight loss (Table 1/II). This study shows that compared to EGII, more EGI is required to obtain similar improvement in pilling resistance, indicating a different mode of action of these endoglucanases. Thus, it seems that the cellulase action can be directed towards the fibre ends or fibrils, readily forming pills by selecting a suitable cellulase composition.

3.2.1 Effect of low EG ratios of cellulase mixtures on cotton interlock properties (unpublished data)

The most promising monocomponent cellulase mixtures with respect to improved pilling resistance and reduced strength loss, i.e. EGI/CBHI and EGII/CBHI, were further tested using different enzyme ratios, i.e. 5/95, 15/85 and 35/65. Treatments were carried out using a total enzyme dosage of 5 mg protein/g knitted cotton fabric for 2 hours. No additional mechanical action was used in the Linitest treatments. The fabric used in these tests was bleached before the treatments. The results of the tests are listed in Table 13.

Table 13. Weight loss, liberation of reducing sugars, strength loss and pilling values of the knitted cotton fabric treated in the Linitest with different cellulase mixtures.

Sample	Weight loss % of d.w.	DNS % of d.w.	Strength loss %	Pilling value
Ref.	0.11	0.04	-	2–3
CBHI 100%	0.23	0.33	3.8	3–4
EGI/CBHI 5/95 %	1.47	1.09	4.7	5
EGI/CBHI 15/85 %	1.45	1.19	4.8	5
EGI/CBHI 25/75 %	1.63	1.18	8.6	4
EGI/CBHI 35/65 %	1.27	1.21	9.8	4–5
EGI/CBHI 50/50 %	2.07	1.06	8.5	4–5
EGI 100%	0.2	0.29	2.1	4
EGII/CBHI 5/95 %	0.75	0.76	8.5	5
EGII/CBHI 15/85 %	0.88	0.78	6.4	5
EGII/CBHI 25/75 %	1.09	0.69	9.0	5
EGII/CBHI 35/65 %	0.88	0.74	9.0	5
EGII/CBHI 50/50 %	0.99	0.57	9.2	4–5
EGII 100%	0.47	0.23	6.0	4–5

The synergistic effect was clearly visualized in the increased weight loss even when low amounts of endoglucanases were combined with CBHI. Even the lowest ratios of endoglucanases in the mixtures improved the pilling properties of the interlock fabric. Similar observations were reported by Reinikainen (1994) with β-glucan as a substrate. In his study even a small amount of EGII combined with CBHII was able to decrease the viscosity of β-glucan.

3.2.2 Experimental cellulase mixtures of *T. reesei* cellulases in finishing of cotton fabrics (IV, unpublished data)

Crude cellulase preparations have mainly been used in industrial biofinishing and denim processes. The commercial cellulase preparations usually also contain buffers, surfactants and preservatives.

In this part of the work, different cellulase mixtures produced by Röhm Enzyme Finland (currently AB Enzymes) were tested on cotton knitted (IV and unpublished data) and woven fabrics (IV). The experimental cellulase mixtures A–F with different cellulase profiles were produced by genetically modified *T. reesei* strains. The cellulase profiles in each mixture are presented in Table 9. Purified EGII, being the most effective cellulase in the prevention of pilling both alone and in defined mixtures as discussed in papers I and II, was used as a reference in the treatments.

The data obtained with different experimental mixtures showed, that Cellulase F, an EGII-enriched preparation, gave the highest depilling value with the lowest strength loss on knitted fabric (Table III / IV). However, purified EGII alone was even more efficient with respect to good depilling with minimal negative effect on the knitted fabric.

When ring-spun fabric was treated, the best depilling value was again obtained with EGII alone with the lowest weight loss (Tables II and IV/ IV). In the case of mixtures, Cellulase B, containing EGII and CBHI, reduced the pilling tendency of both ring-spun and open end-spun fabrics. For open end-spun fabrics Cellulase E, an EGII-enriched / CBHI-negative preparation, was the best preparation for reducing pilling. Thus, the high pilling removal efficiency was dependent on the fabric type.

It was clearly shown in this study that monocomponent EGII and EGII-based cellulase mixtures gave the most positive depilling results. This is in agreement with the findings of Miettinen-Oinonen *et al.* (1997b) with *T. reesei* cellulase compositions of elevated EGII contents.

To confirm the results, the effects of smaller enzyme dosages, i.e. 0.01–5.0 mg of protein per gram of substrate, of experimental mixtures E and F and

Table 14. Effects of different dosages of EGII, Cellulase E, Cellulase F and Biotouch L on cotton interlock properties (unpublished data).

Sample	Dosage mg/g	Weight loss %	DNS % of d.w.	Strength loss %	Pilling value
EGII	5	1.9 (+/- 0)	0.27	13.7	5
	2.5	1.4 (+/- 0.1)	0.20	5.4	5
	1.0	1.0 (+/- 0)	0.17	6.4	5
	0.5	0.7 (+/- 0)	0.13	1.4	5
	0.25	0.5 (+/- 0.1)	0.10	4.6	4
	0.1	0.4 (+/- 0)	0.07	2.4	4
	0.01	0.4 (+/- 0.1)	0.04	1.8	3–4
Biotouch L	5	2.9 (+/- 0.2)	1.53	8.9	5
	1.0	1.1 (+/- 0.1)	0.71	8.2	4
	0.5	0.9 (+/- 0)	0.47	5.5	4
	0.25	0.6 (+/- 0.1)	0.28	4.0	3–4
	0.1	0.6 (+/- 0.1)	0.19	4.3	3–4
	0.01	0.4 (+/- 0.1)	0.05	3.7	2–3
Cellulase E	1.0	1.4 (+/- 0.3)	0.44	6.2	5
	0.5	1.2 (+/- 0.3)	0.32	7.5	5
	0.25	0.7 (+/- 0)	0.22	7.9	4–5
	0.1	0.6 (+/- 0.1)	0.14	7.2	3–4
	0.01	0.5 (+/- 0.1)	0.04	3.3	2–3
Cellulase F	1.0	1.2 (+/- 0.1)	0.68	5.9	5
	0.5	0.7 (+/- 0.1)	0.47	4.9	5
	0.25	0.6 (+/- 0)	0.30	10.3	4
	0.1	0.4 (+/- 0.1)	0.16	7.2	3–4
	0.01	0.3 (+/- 0.1)	0.04	0.6	2–3
Ref.	-	0.2 (+/- 0.1)	0.04	-	2–3

commercial cellulase Biotouch L were evaluated on the cotton interlock fabric (unpublished data, fabric and treatment conditions as in paper IV). The results obtained in these measurements are shown in Table 14. When the lowest enzyme dosage was used, the strength loss caused by Cellulase F was the lowest 0.6%, whereas monocomponent EGII caused 1.8% and cellulase E 3.3% strength loss. On the other hand with this dosage Cellulases E and F and Biotouch L did not improve the cotton pilling.

In one hour treatments even the lowest dosage, i.e. 0.01 mg/g of EGII was sufficient to improve the pilling properties of knitted fabric. Low EGII dosages reduced the strength of knitted fabric less than the same dosages of Biotouch L and Cellulase E.

Cortez *et al.* (2002) and Bishop *et al.* (2001) used the same fabrics and partly the same cellulose preparations to evaluate the effects of cellulases on dimensional stability after pad-batch treatment. Their results revealed that greater improvements in dimensional stability were obtained with cellulase F, with lower strength loss compared to Biotouch L treatments. Many other studies and patents of different *T. reesei* derivatives such as enriched or deleted preparations have also been reported, but usually no detailed compositions have been given, see also Table 6. Comparisons between the endotype and exotype cellulases have been made, although the dosages of enzymes cannot be compared.

Thus by tailoring the amounts and types of different cellulases in the enzyme mixture, the positive effects obtained with enzymes, i.e. reduced pilling tendency, fabric hand properties and dimensional stability can be maximized with minimum strength and weight losses.

4. Conclusions

Cellulase enzymes are important tools in the textile industry for processing cellulose fibres. They provide an economical and ecological way to treat cotton and cotton-containing fabrics. Although cellulases have been used for biostoning and biofinishing cotton since the 1980s, many crude mixtures of cellulases are still used in textile processes today. A common problem associated with treatments with crude mixtures is that the treated fabrics exhibit significant strength and weight losses. This study has been among the first attempts to study the effects of individual cellulases and their synergistic action on cotton fabric properties.

The results obtained provide new information on the effects of purified individual cellulases on cotton fabrics. Purified EGII cellulase was found to be the most effective single cellulase in preventing pilling. It was also shown that even a very small amount of EGII improves pilling values without any significant strength or weight reduction, and thus no correlations between good pilling values and strength or weight loss were observed with purified EGII. Furthermore, it was shown that EGII is the most effective of the main cellulases at removing colour from denim fabrics. Mechanical action had a significant impact on EGII treatments. Larger scale tests with different mechanical agitation levels and treatment parameters would provide more information about the interaction of cellulases and cotton.

In addition to mono-component cellulases, the results obtained with defined cellulase mixtures provided useful knowledge for designing new production strains of *T. reesei*. The EGII-based cellulase mixtures gave the most positive depilling results and stone washing effects. Furthermore, it was confirmed with purified cellulases that the technical properties resulting from the enzymatic treatment of cotton fabrics depend not only on the type and concentration of enzyme used, but also on the type of fabric. Thus, for optimal biofinishing performance, an enzyme product should be selected on the basis of cellulase selectivity, existing equipment, fibre and fabric type.

Better understanding of the mechanism of enzymatic hydrolysis of cotton, e.g. the difference between EGI and EGII adsoption phenomena, is still required to be able to exploit the enzymes in textile processes in order to achieve the desired finishing effect with minimum negative impact.

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